

ANATOMICAL AND HISTOCHEMICAL STUDIES  
OF THE ACTION OF THE  
HERBICIDES - TORDON 50D and TORDON 22K

by

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July 1972

This thesis is submitted in fulfillment  
of the requirements for the degree of  
Doctor of Philosophy in the Australian  
National University.

Except where otherwise acknowledged, the work described in this thesis is original and entirely my own.

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## ACKNOWLEDGEMENTS

This project was carried out during tenure of an Australian National University Research Scholarship. I wish to express my thanks to the university for financing this project and to Professor J.D. Ovington for permitting me to study in the Department of Forestry.

Most sincere appreciation is extended to my supervisor, Dr. E.P. Bachelard. For his criticism, constant encouragement, and gentle guidance, I am ever grateful. I also wish to thank Dr. W. Heather of the Department of Forestry, who took time out from a busy schedule to help in the resubmission of the thesis while Dr. Bachelard was on sabbatical leave.

Dr. C. Greenham, formerly of C.S.I.R.O., Canberra, was most helpful with the alternating-current bridge experiments reported in Chapter 5 and with the interpretation of the results.

Appreciation is also given to Dr. E.G. Brittain of the Botany Department, A.N.U., for making available the facilities of his Electron Microscope Laboratory and for permitting the extensive use of the electron microscope.

The assistance and guidance of Mrs. A. Peterson in preparing material for electron microscope study are greatly appreciated. Her friendly encouragement is recorded here with thanks.

I am grateful for the advice of Dr. M. Tanton with regard to the presentation of photographic material and for the extensive use of his darkroom facilities.

Finally my sincere thanks to Mrs. Driver for typing the figure headings for Book 2. Warm appreciation is also extended to Miss Robyn Glen for her patience and perseverance in typing the entirety of Book 1.

## PREFACE

This thesis has been re-written following criticisms of the original submission by the examiners. In the original, the examiners felt that the basis for the study was not clearly stated and that the choice of concentrations and herbicides used were not fully justified.

New work has been included on preliminary studies to justify the subsequent biological examination and the conditions under which it was made. The results of this new work are presented in Chapter 2. The tool for the examination of herbicide action is the differential effect of the commercial herbicide formulations. This differential effect is supported by the results of Chapter 2 and also reports of the field work of Bachelard and co-workers (Bachelard et al. 1965, Bachelard and Boughton 1967, Bachelard and Johnson 1969). Factors determining selectivity and possibly affecting this differential effect are discussed in detail in Chapter 1, section 1.

A major criticism of the technique used in the nucleic acid study resulted in a fresh examination of the herbicide effects on ribonucleic acid using a modified histochemical technique (Dickinson and Heslop-Harrison 1970). The problems associated with accurate nucleic acid assessment in plant tissues have also been discussed in detail. The photographs presented in this section have been limited to significant portions of the period of study.

The final discussion of the results of this study (Chapter 8) was criticised for its undue reference to animal membranes at the expense of relevant literature on plant membranes. To a certain extent this criticism will be satisfied by the inclusion of further reports from the literature dealing with plant membranes, particularly those of



van Stevenick (1965), Smith (1970), and Higinbotham et al. (1970). However it must be emphasized that an explanation of the unregulated cell growth exhibited in root tissues in this study may still be related to those findings of workers at the forefront of cancer research.

The re-writing of this thesis has emphasized its original contributions. The photographs have been retaken and enlarged to demonstrate the features of the text. In addition to the photographs included in the original thesis, many new photographs have been included to show replicate material and to further support the argument.

## ABSTRACT

The herbicides, picloram (4-amino-3,5,6-trichloropicolinic acid) supplied as Tordon 22K, and Tordon 50D (1 part picloram ; 4 parts 2,4-D) were examined for their effects on Pinus radiata, D. Don and Eucalyptus tissues in an attempt to determine a primary site of herbicide action.

In preliminary studies a range of concentrations of both pure and commercial herbicide formulations was used. The differential effects of Tordon 22K and Tordon 50D on P. radiata tissues were not fully explicable by a particular herbicide property. The responses of tissues to suitable concentrations of commercial formulations of Tordon 22K and Tordon 50D were investigated.

The herbicides applied, at 25ppm Tordon 22K and 25ppm Tordon 50D (picloram : 2,4-D, 1:4), affected the subsequent development but not germination of seeds of P. radiata. Herbicides in direct contact with germinated seeds severely checked root elongation, caused swelling of both the root and the hypocotyl and cotyledons of most developing seedlings were flaccid in appearance and reduced in length.

Both herbicides induced irregularities in the plane of cell division. Rapid, apparently uncontrolled cell division resulted in a layer of cell multiplication in the roots of developing seedlings. This zone of cell proliferation was largely responsible for the swelling of the root but, with Tordon 22K-treated tissues, irregular expansion of the cortical cells also contributed to this swelling. Abnormal swelling of the shoot apices of seedlings from herbicide-treated seeds was caused by changes in the plane of cell division, and increased number of divisions. The elongation of individual cells was reduced especially in root tissues.

Herbicides appeared to stimulate the onset of cell division in the pith and cortical cells in the hypocotyl of developing P. radiata seeds, and to inhibit the elongation of these dividing cells. There was also an early maturation of cells, and an apparent disruption of cellular integrity.

Qualitative histochemical examination comparisons were made of storage protein, carbohydrate, lipid, and ribonucleic acid contents of herbicide-treated and control tissue. Apparently the demand for high energy products of starch and lipid catabolism was reduced, possibly because of decreased vigor of the treated seedlings. Cytoplasmic-RNA levels seemed to be greater in herbicide-treated than in untreated root tissues. A significant increase in the size of nucleoli occurred in root cells of P. radiata seedlings treated with both herbicides; this hypertrophy may have indicated a physical effect of the herbicides, or possibly increased DNA synthesis.

To this stage, both Tordon 22K and Tordon 50D had similar effects on the tissues examined but these herbicides have previously been shown to have differential effects on P. radiata seedling material. Effects of the herbicides on leaf and needle segments of Eucalyptus and P. radiata following in vitro treatment were examined. Tordon 50D had a very rapid effect on membrane permeability in cells of P. radiata needle segments; Tordon 22K had no such effects. These responses were observed initially using the light microscope, and were confirmed using an alternating-current bridge with which currents of low and of high frequency were passed through the tissues. Effects of Tordon 50D were largely due to its 2,4-D content.

Both Tordon 50D and Tordon 22K had similar effects on leaf discs of eucalypt tissues with Tordon 50D being more rapid in its action. Cytoplasmic membranes were ruptured,

and the integrity of the chloroplasts was also destroyed, resulting in the disruption of their ordered arrangement within the cells and in some cases, the release of their contents.

These effects of herbicides on membranes and organelles in eucalypt and P. radiata tissues were confirmed and extended by use of the electron microscope. Additionally, the response of isolated tissue segments to herbicides was largely confirmed in intact seedlings when the pure herbicides, picloram and 2,4-D, were used.

On the basis of results obtained, and from recent reports in the literature, it is argued that plant cell membranes could be a primary site at which herbicides act.



## TABLE OF CONTENTS

TITLE PAGE	
ORIGINALITY OF THESIS	i
ACKNOWLEDGEMENTS	ii
PREFACE	iii
ABSTRACT	v
TABLE OF CONTENTS	viii
 CHAPTER 1 INTRODUCTION	
1:1 The importance and role of herbicides in forestry	1
1:2 Morphological and anatomical responses to auxin herbicides - a literature review	8
1:2.1 Morphological effects	9
1:2.2 Anatomical effects	12
1:3 Physiological responses by plants to auxin herbicides - a literature review	15
1:3.1 Respiration and metabolism of stored foods	15
1:3.2 Photosynthesis	18
1:3.3 Oxidative-phosphorylation responses	20
1:3.4 Protein and amino acid synthesis	21
1:3.5 Enzyme systems	22
1:3.6 Nucleic acid metabolism	23
1:4 Purpose of present study	27
 CHAPTER 2 PRELIMINARY STUDIES OF HERBICIDE ACTIVITY	
Experiment 1 Effects of concentration of pure and commercial herbicide formulations on <u>P. radiata</u> seed germination and early seedling growth.	29
Experiment 2 Effects of the triisopropanolamine and potassium salts of picloram on <u>P. radiata</u> and eucalyptus seedlings.	32

Experiment 3	Effects of concentration of pure and commercial herbicide formulations on <u>P. radiata</u> and eucalyptus seedlings.	34
Conclusions		38
CHAPTER 3	ANATOMY OF GERMINATING SEEDS	
3:1	Materials and methods	42
3:2	Results	
3:2.1	Germination and gross morphology	43
3:2.2	Measurements of cell lengths	45
	(i) Pith cells	
	(ii) Cortical cells	
	(iii) Cotyledon cells	
3:2.3	Shoot apical meristem	52
3:2.4	The radicle and root meristem	54
3:3	Discussion	58
CHAPTER 4	MICROSCOPIC HISTOCHEMISTRY OF GERMINATING SEEDS	
4:1	Materials and methods	66
4:1.1	Histochemical procedures	67
4:2	Results and observations	69
4:2.1	Proteins	
4:2.2	Carbohydrates	
4:2.3	Lipids	
4:2.4	Ribonucleic acid	
4:3	Summary	86
CHAPTER 5	EFFECTS OF HERBICIDES ON LEAF AND NEEDLE TISSUE	
5:1	Materials and methods	88
5:2	Results and discussion	89

CHAPTER 6	ULTRA-STRUCTURAL CHANGES IN LEAF AND NEEDLE SEGMENTS	
6:1	Materials and methods	94
6:2	<u>Pinus radiata</u> needle segments	96
6:2.1	Effects on nuclear and plasma membranes	96
6:2.2	Effects on chloroplast structures	98
6:3	<u>Eucalyptus viminalis</u> leaf discs	100
6:4	Discussion	102
CHAPTER 7	EFFECTS OF HERBICIDES ON INTACT SEEDLINGS	
7:1	Materials and methods	109
7:2	Results	
7:2.1	Gross morphology	110
7:2.2	Anatomical effects on <u>P. radiata</u> tissues	111
7:2.3	Anatomical effects on <u>E. camaldulensis</u> tissues	113
7:3	Discussion	114
CHAPTER 8	DISCUSSION	119
REFERENCES		137

## CHAPTER 1

### INTRODUCTION

#### 1:1 The Importance and Role of Herbicides in Forestry

The use of inorganic chemicals to eradicate unwanted herbaceous and woody vegetation had been developed on an extensive scale in the forests of Australia and India as early as 1915 (Sutton 1958) but it was only in the early 1940's that intensive research on the problems of chemical weed control was stimulated by the discovery of the auxin-like properties of the phenoxyacetic acids, and subsequently, the herbicidal action of 2,4-dichlorophenoxyacetic acid (Freed 1961). Although early research on chemical weed control was limited primarily to agricultural crops, herbicides for forestry use have emerged as one of the most versatile and potentially useful tools in forest management.

The development of herbicides for forestry purposes is due partly to the high cost of alternate methods of weed control as a result of rising labour costs, and partly to an intensification of forest management coupled with increased investment in forest land (Sutton 1958). In order to maintain or increase forest productivity, while at the same time controlling costs, the forest land manager is obliged to employ the most economical silvicultural tool available to him. Herbicides very often fill this role in weed control.

In some instances, where labour is cheap and time not of the essence, other means of removing unwanted vegetation may be employed, but in order to attain the desired degree of weed control, herbicides must often be used in conjunction with other methods (Jaciw 1968).



The release of planted or naturally-occurring species in a stand, usually conifers, from competing or over-topping vegetation, and the preparation of sites prior to planting sometimes requires the use of chemicals for weed control (MacArthur 1969). Herbicides are often used to kill large, unmerchantable, or damaged trees after a commercial cutting operation or, sometimes in fire damaged stands. Herbicides are also utilized in forest tree nurseries, an area of intensive management, throughout the life of the crop.

Although considerable progress has been made in the development of new and more effective herbicides for forestry purposes, more fundamental and applied research is still needed before herbicides can be used confidently. Inconsistent results are frequently obtained, due largely to deficiencies in our knowledge of the physiological and environmental factors affecting herbicide action.

Understanding the fundamental nature of herbicide action could be an aid in determining herbicide selectivity. A herbicide which permits differential treatment between crop and weed has been defined as a chemical at a certain concentration used in a particular manner to kill or check the growth of weeds in a growing crop without damaging the crop (Sutton 1967). In forestry practice, the unwanted vegetation must often be treated in the presence of the crop species, and for maximum benefit, the herbicide should remove only unwanted vegetation without affecting the growth and development of the crop species. Understanding herbicide selectivity will permit a highly specific use of herbicides in forest management. It will allow the release of individual crop species, either conifers or hardwoods, from competing vegetation, and will enable the forest manager to control stand composition to maintain an economic balance.

Selectivity however, is not an absolute herbicide

property. Under certain conditions, a herbicide which is regarded as toxic to hardwoods but no conifers, may be extremely toxic to the conifers (Kozlowski and Sasaki 1968a). Selectivity is relative and can be achieved to a degree only when numerous variables have been considered.

Manner of herbicide application may affect the degree of selectivity attained. Sutton (1967) notes that herbicides regarded as non-selective may sometimes be used selectively by carefully applying the herbicide to the unwanted vegetation and leaving the crop untreated. The concentration of herbicide used is also critical in demonstrating selectivity and often the best concentration for field use is that which provides the maximum differential in selectivity between crop and weeds. The herbicide selectivity of the compounds 2,4-D and 2,4,5-T (2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid) is dependant to a large extent on the dosage applied (Sutton 1958). These growth-regulator type herbicides follow the dosage response curve of the natural occurring growth regulator, IAA (3-indole acetic acid) in that low concentrations increase growth while higher concentrations cause growth inhibition and/or death (King 1966). The basis of their selectivity is the susceptibility of certain plants to particular herbicide concentrations.

The chemical nature of the herbicide molecule also influences selectivity. This largely affects the level of herbicide absorbed into the plant. This appears to be essentially a surface phenomenon and the use of surfactants or wetting agents with a herbicide formulation enhances absorption particularly in foliage treatments (Klingman 1961). As well as the chemical nature of the herbicide, physical properties of the spray itself may influence selectivity. The volume of spray retained on the plant may determine the

dosage of herbicide received. Apart from differences in morphology and the nature of plant surfaces, spray volume retained by the plant may be affected by droplet size and volume rate of the herbicide applied (Holly 1964).

By choice of herbicide, dosage and manner of application, it may be possible to obtain a degree of selectivity between crop species and unwanted vegetation. However, in understanding herbicide action, the reason for selectivity is as important as how it is achieved. The basis of herbicide selectivity may lie in physical, physiological, or biochemical differences in the species treated (Holly 1964). For the most effective and economical use of herbicides, it is important to know which of these causes is operative in a particular situation.

Physical factors affecting selectivity are usually related to differences in the structure and growth habits of plants. A herbicide might be toxic to both the crop and weed species given equal ease of entry into both plants. However, the two may vary in susceptibility because of physical differences such as the nature of the bark, leaf or needle surface, or life form (Sutton 1967). Differences in herbicide retention and penetration are often due to differences in plant morphology. For example, the greater susceptibility of hardwoods than of hardened-off conifers to 2,4-D treatments is largely a consequence of the broader, hairy leaves of hardwoods in contrast to the often thick-cuticled, narrow needles of conifers (Sutton 1967, Kozlowski and Sasaki 1968a).

Physiological factors influencing herbicide selectivity are often related to physical factors and depend on the state of plant growth. When conifer shoots are expanding, they are very susceptible to 2,4-D while hardened-off growth is resistant (Kozlowski and Sasaki 1968a).

Sutton (1967) notes that the herbicide, amino-triazole, is highly toxic to the early spring growth of Picea glauca (Moench) Voss but can be used safely on the species from mid-summer onwards. Differences in translocation of the herbicide may also affect selectivity (Holly 1964). The movement of certain chemicals from the leaves is associated with the flow of sugars, produced in photosynthesis, usually towards sites of high metabolic activity (Wain 1964) and movement is therefore slight in plants kept in the dark or in a low CO<sub>2</sub> atmosphere. By contrast, actively growing plants have been found to translocate 2,4-D rapidly (Klingman 1966). Selectivity may also result from the different root volumes of plants at the time of treatment. Older, better established species with greater root volumes may be more susceptible to certain soil-applied herbicides than newly planted stock with roots occupying smaller volumes of soil (Sutton 1967). Herbicides such as fenuron (N,N-dimethyl-N'-phenyl urea) which are applied in pellet form and penetrate the soil should therefore be introduced to the forest area as soon as possible after planting to ensure that selectivity between well-established, unwanted vegetation and newly-rooted species is achieved (Sutton 1967).

The cause of differences in herbicide response between crop and weed species which are morphologically and physiologically similar may be difficult to find. The removal of one conifer species from a mixed stand without inflicting damage to a selected conifer crop becomes a very subtle matter and often depends on biochemical selectivity between plants. Biochemical selectivity may have more than one mode of action. It may be due to differential accumulation of the chemical within the plants or to differential breakdown of the chemical within tissues between species (King 1966).



Wain (1964) states that the toxicity of a herbicide to a particular species may be limited by absorption of the herbicide molecules to sites not concerned in physiological response or the molecules may become associated with certain enzyme systems where they are converted into compounds with little or no toxicity. The biochemical selectivity of some herbicides may lie in the different enzyme systems of plants (Sutton 1958). Some enzyme systems may either detoxify a harmful chemical or may make a non-toxic chemical toxic. The differences in susceptibility of certain plants to 2,4-D may be related to the possession of particular enzymes with the ability to oxidize the carboxyl and methyl carbons from the 2,4-D side chain and render the molecule non-toxic (Crafts 1964). Herbicide selectivity of simazine is mainly the result of differential breakdown by enzymes within plants (Wain 1964). Enzyme systems of resistant plants such as corn are able to metabolize simazine fairly rapidly and convert it to a non-toxic form, while susceptible plants, lacking these particular enzyme systems, accumulate the herbicide at toxic levels. Some plants may also possess enzymes that convert a non-toxic chemical to a toxic form. The phenoxybutyric acid compounds have weed killing properties because susceptible plants have enzymes capable of degrading the non-toxic butyric forms to the toxic acetic acid compounds (Wain 1964). For example, 2,4-DB (2,4-dichlorophenoxybutyric acid) will remain unchanged in some plants while in others it will become converted by certain enzyme systems to 2,4-D. Selectivity of the biochemical type obviously depends upon plant metabolism but the rate of action can be influenced by other factors such as the age of the plants and air temperature.

Biochemical selectivity may also be due to a selective effect on the protoplasm of cells involving membrane permeability, variations in chemical constituents of the protoplasm, differences in response to pH changes, or to some

other response of cell metabolism to the presence of the herbicide (Klingman 1961).

Differences in dosage, herbicide ingredient and manner of application as well the physical, physiological or biochemical differences between plants are factors that will affect herbicide selectivity. These factors may operate separately or in unison to give a wide range of effects from total vegetation kill to selective weed control. However, too little is known at present about either the modes of action of herbicides or of the biochemical systems on which they act to accurately explain selectivity.

The most effective herbicides for the long term control of unwanted woody vegetation are 2,4-D, 2,4,5-T and picloram (4-amino-3,5,6-trichloropicolinic acid). The auxin-like properties of 2,4-D and 2,4,5-T have long been known (Brian 1964) and Kefford and Caso (1966) and Eisinger et al. (1968) have recently shown the auxin activity of picloram. Mixtures of picloram and 2,4-D have been developed to broaden the effectiveness and reduce the cost of treatment of mixed populations of weeds in growing crops. The use of herbicide mixtures may effectively control more species, may reduce the overall rate of herbicide applied and hence the cost of treatment, and may also reduce partially herbicide residues by substituting effective herbicides with shorter residual characteristics (Bovey et al. 1968). Picloram was first introduced for evaluation purposes in 1963 and effective control of weeds has been achieved using picloram alone (Alley 1967). However, factors of high soil residue and cost of picloram prompted the development of mixtures of picloram and 2,4-D, notably Tordon 50D (a mixture of 1 part picloram and 4 parts 2,4-D) in an attempt to reduce the picloram dosage applied and still obtain the desired results. Mixtures of picloram and 2,4-D appear to have distinct advantages but

knowledge of their interactions is still very vague (Bovey et al. 1968).

Although these herbicides and their mixtures are most effective in killing some species in some areas at some times, their success can rarely be guaranteed. Much more must be known both of the physiological and environmental factors affecting their activity and of their biochemical mode of action before the potential of these herbicides can be fully realised.

## 1:2 Morphological and Anatomical Responses to Auxin

### Herbicides - a literature review

Endogenous growth hormones such as the indole auxins (3-indole acetic acid, indole pyruvic acid, and possibly indole acetole nitrile) control the biochemical environment of plant tissues and ultimately determine their physiological fate. The introduction of synthetic growth regulators into this delicately balanced system upsets the normal functioning of the tissues and causes many and varied responses (van Overbeek 1964).

Chlorophenoxyacetic acids may stimulate or inhibit plant development depending on their concentration, and can produce disturbances in the growth of nearly all tissues (Kiermayer 1964). These auxin herbicides affect young cells that are still growing actively, particularly those of the embryonic and meristematic regions, but relatively mature cells are less affected directly (Crafts 1961, Muzik 1970). Picloram (4-amino-3,5,6-trichloropicolinic acid) can also be classed as an auxin herbicide, having the minimal structural requirements for auxin activity (Eisinger et al. 1968), and Goodin et al. (1966) reported that picloram is freely translocated throughout the plant and is readily absorbed by

both leaves and roots. It is particularly effective against most woody species as well as herbaceous broad-leaved plants, and growth responses following picloram application resemble those resulting from treatment with the chlorophenoxyacetic acids. According to Muzik (1970), any plant sensitive to 2,4-D will generally be affected by picloram.

A survey of the literature pertaining to 2,4-D and to picloram will help point to the similarities in the morphological and anatomical plant responses to these growth regulators.

### 1:2.1 Morphological Effects

One of the earliest reports on plant response to 2,4-D was made by Nutman and co-workers in 1945. Working with red clover seedlings grown in soils treated with 1 part per million (ppm) and 100 ppm of 2,4-D, they observed that, at both concentrations, the top and root growth of the seedlings was inhibited. The stunted root system was accompanied by thickening of the root cortex and the development of numerous short lateral roots (Nutman et al. 1945). Subsequently, other workers have shown roots to be more sensitive to auxin herbicides than the above-ground portions of the plant. Inhibition of root elongation coupled with increased production of lateral roots, results in a very stunted, branched structure with impaired functions (Gorter and Van der Zweep 1964). Rojas-Garciduenas et al. (1962) found growth of both the plumule and radicle of wheat seedlings to be depressed by 2,4-D but whereas only 5-6 ppm affected the radicle, 10-1000 ppm was required to inhibit growth of the plumule. Bean seedlings treated with 10-1000 ppm 2,4-D showed a marked growth suppression of the shoot and root apices. Roots developed tumor-like growths, stems were abnormally thick, and there was overall necrosis of the seedlings. Wilde (1951), also



using bean plants, found a soil application of only 5 mg of 2,4-D sufficient to severely check root growth, stimulate lateral root production, and cause swelling of the root tips. Wilde concluded that these responses, whether the result of cell enlargement or cell proliferation, were the direct effect of 2,4-D on the normal activity of the root meristem. Kreps and Alley (1967) reported that Canada thistle (Cirsium arvense (L) Snop.) treated with 0.25-1.0 lb/acre of picloram showed severe swelling of the roots and eventual splitting and deterioration of the whole root system. The response of the plants to 2 lbs of 2,4-D was not as dramatic as with picloram but swollen root collars and some internal deterioration of the roots were observed. Whitworth and Muzik (1967) observed swelling of the root tips and a permanent growth inhibition of field bindweed (Convolvulus arvensis L.) following foliar application of 2,4-D. 2,4-D appeared to stop root elongation by arresting cell division and growth in the region of the apical initials.

Auxin herbicides have similar effects on roots of conifers as for angiosperms. Fromantin (1958) examined the action of two auxin compounds, 2,4,5-trichlorophenoxyacetic acid and indole acetic acid on several conifers and found a general inhibition of hypocotyl and root development and, with 2,4,5-T, a swelling of these organs.

Root growth of Pinus resinosa Ait. seedlings was depressed and high shoot/root ratios were obtained after treatment with 1 ppm 2,4-D (Kozlowski and Sasaki 1968b). The high shoot/root ratios were due both to root growth depression and shoot growth stimulation. Extremely low concentrations (0.001-1.0 ppm) of both 2,4-D and picloram inhibited root elongation and induced morphogenetic changes in the cotyledons. Higher concentrations of both herbicides were necessary to suppress shoot elongation. Johnson (1967) found Pinus radiata D. Don seedlings from soil treated with 0.2-0.5 lb/acre of picloram

had fewer fine roots compared with controls, and had a poorly ramified root system. Root development and elongation was severely limited by picloram and the hypocotyls of treated seedlings were distinctly thicker than in control seedlings.

Although the above-ground portions of a plant are somewhat more resistant to external auxin application than the root system, they are nevertheless affected to varying degrees depending on the herbicide concentration and the nature of the plant. Several workers have shown that picloram causes twisting and distortion of stems and leaves in susceptible broad-leaved plants similar to the response caused by the phenoxyacetic acids (Hamaker et al. 1963; Eisinger et al. 1968). Arnold and Santelmann (1966) reported bending of the terminal portions of several broad-leaved species after treatment with picloram. Leaves were cupped and curled, resembling the symptoms of drought stress, and stems were extremely swollen in the apical region.

Sasaki and Kozlowski (1968 a) systematically examined the effect of herbicides on the development of Pinus resinosa from seed germination to the cotyledon stage. Germination was not significantly affected by 2,4-D concentrations up to 100 ppm but subsequent shoot development was altered. Seedlings developed swollen stems and abnormally shrivelled, chlorotic cotyledons. Similar effects were noticed when seedlings of P. resinosa were exposed to 1 ppm of picloram (Kozlowski and Sasaki 1968 b).

Pinus radiata seedlings show similar morphological responses after treatment with picloram and the chlorophenoxyacetic acid herbicides. In soil applications, 2,4,5-T and picloram both caused stem curvature in germinating seedlings, and the tangling and curling of cotyledons (Johnson 1967). When applied to the tips of one-year-old P. radiata seedlings, 2,4,5-T and two formulations of picloram caused

varying degrees of injury to the plants. One formulation of picloram, Tordon 50D (1 part triisopropanolamine salt of picloram + 4 parts 2,4-D) inhibited shoot growth and killed the tips of seedlings, effects similar to those produced by 2,4,5-T. The other picloram formulation, Tordon 22K (potassium salt of picolinic acid) had no effect on shoot extension but caused swelling of the apex, and stimulated lateral branching close to the apex (Bachelard and Boughton 1967).

### 1:2.2 Anatomical Effects

The response of the roots and shoot apical regions of a plant to external auxin supplies appears due to an effect on meristematic tissues. Abnormal cambial activity is stimulated by auxin herbicides (Gorter and Van der Zweep 1964), and Eames (1949, 1950) suggested that internal modifications produced by 2,4-D in bean seedlings were initiated in immature tissues or in tissues that, although mature, readily became meristematic. Normal cells and tissues in the primary phloem of bean hypocotyls were supplanted by fleshy parenchyma cells. These thin-walled cells proliferated freely, disrupted phloem elements and eventually crushed companion cells and smaller sieve tubes. The cell proliferation was similar to uncontrolled cambial activity, adding many new cells just outside the provascular core. This caused extreme swelling of the hypocotyls with eventual compression and rupture of outer tissues. Chrispeels and Hanson (1962) found that stimulatory levels of 2,4-D caused a reversion of some plant tissues back to a meristematic state. This was especially evident in stem sections, where vascular parenchyma became meristematic. The new meristematic zone led to subsequent tissue proliferation and eventual crushing of the phloem cells.

A possible mechanism of the herbicidal effect of 2,4-D is tissue proliferation interfering with the normal translocation of food. Various workers have suggested similar activity by picloram. Roots of Canada thistle showed distortion of the cortical tissue, and disintegration of the cambial and phloem elements two weeks after the plants had been sprayed with 0.25 lbs/ac of picloram applied as Tordon 22K (Lee et al. 1967). Kreps and Alley (1967) also using Canada thistle, reported parenchyma cells in the centre of the cortex were partially or completely ruptured and, in severe cases of picloram activity, the cambium and phloem were totally destroyed, leaving only the central xylem and periderm intact. Picloram treatments were 0.25, 0.5 and 1.0 lbs/ac.

Increased cell division of the procambium of ironweed (Vernonia baldwini Torr.) leaves sprayed with 0.25 and 0.5 lbs/ac of picloram eventually obliterated all primary phloem tissue. Phloem parenchyma, sieve tubes, and companion cells were disintegrated, and it was concluded that the stimulation of procambial activity leading to the destruction of the phloem probably accounted for the rapid herbicidal action of picloram (Scifres and McCarty 1968).

Fisher et al. (1968) found that cambial initials of Phaseolus vulgaris L. seedlings treated with picloram first divided normally in a periclinal direction but later divisions were anticlinal (at approximately right angles to the outer surface), ultimately producing adventitious roots which pushed through the cortical tissues stretching the outer epidermal cells until fissures developed. Root initials were also initiated in the leaves by the same process. Johanson and Muzik (1960) reported abnormal cell division in the pericycle and endodermis in roots of 2,4-D treated wheat plants which led to the formation of lateral root primordia.

Weintraub (1953) suggested that the normal polarization (normal orientation) of dividing cells may be disturbed by

growth regulators, resulting in abnormalities and cell proliferation. Changes in the plane of cell division, especially in root tips after treatment with growth regulating substances have also been noted by Kiermayer (1964). Excessive cell division in the outer phloem of field bindweed stems treated with 0.5 ppm 2,4-D was reported to result in the rupture and fragmentation of much of the tissue external to the cortex. Swelling of the root tips was also accomplished by accelerated cell division in the endodermis and pericycle (Whitworth and Muzik 1967). Bradley et al. (1968) observed that the increased leaf thickness of mature apricot leaves sprayed with 50-200 ppm 2,4,5-T was due to stimulated cell division in the palisade mesophyll layer and not to elongation or swelling of these cells. In radicles of soybean seedlings treated with 1.0 lb/ac 2,4-D, Rojas-Garciduenas and Kommedahl (1958) found the total number of cells to be much greater compared with the controls after eighty-five hours as a direct result of stimulated cell division. The average cell length was less in 2,4-D-treated radicles and the authors concluded that the herbicide had reduced cell elongation as well as increased cell division.

One of the most striking effects, therefore, of the chlorophenoxyacetic acid herbicides and picloram, is the rapid and aberrant division of meristematic tissue and subsequent proliferation of some cells leading to swollen and split plant portions and growth malformations. Van Overbeek (1964) has stated that the fundamental physiological action of 2,4-D (and picloram) is to cause abnormal plant growth which eventually kills like cancer.

### 1:3 Physiological Responses by Plants to Auxin Herbicides

#### - a literature review

The large number of physiological activities in the plant reported to be affected by chlorophenoxy herbicides indicates that their mode of action is either very non-specific or that they affect a specific step basic to all observed responses. Mann et al. (1965) suggested that any given herbicide used near its lowest effective concentration will affect only one or two metabolic reactions and they describe this as the herbicide's "target area". 2,4-D has been shown by many workers to influence a wide array of metabolic processes. The search for its primary site of action, the target area, becomes difficult when it is realized that the lowest effective concentration may not affect any particular metabolic process that would result in death of the plant. Not all metabolic activities are equally affected by the same concentration, nor are the same processes affected at all stages of growth by all plants or even by the same plant. The interpretation of the responses is complicated by the large number of interacting variables both within the plant and external to it that contribute to the observed effects. Furthermore, a particular response can vary with the time lapsed between herbicide application and response measurement (Penner and Ashton 1966).

#### 1:3.1 Respiration and Metabolism of Stored Foods

One of the most widely reported responses to auxin herbicides is on respiration and subsequent carbohydrate content in the plant. As a general rule, when applied in herbicidal dosages, 2,4-D increases respiration and upsets the balance between synthesis and use of stored food. Storage carbohydrates such as starches are broken down to sugars

(Muzik 1970). Smith et al. (1947) observed that increased respiratory activity of bindweed tissues following 2,4-D application was accompanied by a rapid disappearance of starch granules from the roots. Total sugar content increased initially at the expense of starch in the leaves. Applied to the roots of the common dandelion (Taraxacum officinale), the effect of 2,4-D on respiration varied according to the herbicide concentration employed (Rasmussen 1947).

Respiration rose slightly and then returned to normal in plants treated with 120 ppm 2,4-D. The greatest respiratory increase, at 480 ppm, was maintained as long as the roots remained alive. The highest concentration (1920 ppm) caused a temporary increase in respiration and then a decrease. Kozlowski and Sasaki (1966) also reported a marked suppression in respiration of Pinus resinosa seedlings treated with 4000 ppm of 2,4-D.

As pointed out by Fang et al. (1960), plant respiration may be stimulated or inhibited by 2,4-D depending on the concentration. This is generally accompanied by a qualitative change in carbohydrate content. Other workers support this view. A large percentage of the total available carbohydrates remained in soybean plants treated with 20 ppm of 2,4-D (Wolf et al. 1950) but hemicellulose and reducing sugars increased in treated roots with a related depletion of stored starch. Picloram applied at a rate of 3 lbs/acre increased the total free carbohydrates (reducing sugars) of both field bindweed and Kochia plants 24 hours after spraying (Young and Feltner 1968).

In addition to effects on overall respiration rates, 2,4-D may also influence the pathway of sugar breakdown in plants. The E.M.P. (Embden-Meyerhof-Parnas) glycolytic pathway of anerobic carbohydrate metabolism is normally considered to be the principal route of respiration in higher

plants (Fang et al. 1960, Hilton et al. 1963). Carbohydrate metabolism undergoes a qualitative change as plants develop. The E.M.P. glycolytic pathway is characteristic of young meristematic tissues and as growth and differentiation occurs, the contribution of the pentose phosphate cycle in glucose breakdown increases. Fang et al. (1960) suggested that 2,4-D affected growth of bean stem tissues by increasing glucose utilization which in turn furnished the energy for the synthesis of cellular constituents, and they found a 0.1% 2,4-D concentration to cause a relative increase in the amount of glucose catabolized by the E.M.P. glycolytic pathway.

This result, however, contrasts with others reported. Humphreys and Dugger (1957 a,b) found  $10^{-3}M$  2,4-D greatly increased respiration of seedling root tips but the actual increase, and the effect of 2,4-D on the E.M.P. and pentose phosphate pathways, depended on the food reserves available to the root. In corn and oat roots with or without food reserves, and in pea roots in the absence of food reserves, 2,4-D increased respiration solely by increased participation of the pentose phosphate pathway. In pea roots supplied with food reserves from the cotyledons, 2,4-D had no real effect on the absolute level of respiration but increased the participation of the pentose phosphate pathway at the expense of the E.M.P. pathway. Reed (1961) also found increased activity of the pentose phosphate pathway over the glycolytic pathway in etiolated sorghum seedlings treated with  $10^{-3}M$  2,4-D. Further work by Black and Humphreys (1962) on etiolated corn seedlings cultured in vitro indicated that a concentration of  $10^{-3}M$  2,4-D decreased the activity of several glycolytic enzymes while those enzymes associated with the pentose phosphate cycle had increased. Inhibition of the E.M.P. glycolytic pathway was reported by Bourke et al. (1962) for root tips of pea seedlings treated with  $10^{-3}M$  2,4-D. No stimulation of the pentose phosphate pathway was observed. They suggest the



divergence of their results from those of Humphreys and Dugger (1957 b) was apparently due to differences in times of treatment and incubation.

These results show that 2,4-D can markedly affect the amount and the pathway of plant respiration but these effects are seemingly not basic to herbicide action since Bourke et al. (1962) showed similar changes to be induced by non-herbicidal analogues of 2,4-D such as 2,6-D and 2,4,6-T.

Lipids (oils and fats) are plant storage products that may be utilized for the production of energy or converted to carbohydrates and hence enter the respiratory pathways in this form (Mayer and Poljakoff-Mayber 1963). Penner and Ashton (1966) noted that reductions in lipid content of 2,4-D-treated plants seemed to parallel the reduction of stored carbohydrates. The conversion or degradation of lipid substances by 2,4-D (50 µg/plant) was reported by Ormrod and Williams (1960), but Mann and Pu (1968) observed a stimulation of lipid synthesis in excised hypocotyls of hemp sesbania at 2,4-D and picloram concentrations of between 1 and 20 ppm.

The depletion of carbohydrate and lipid contents of some plant tissues after auxin herbicide treatment is due to either a diminished production or increased utilization via glucose metabolic pathways. This is apparently not the principal cause of death. As Smith et al. (1947) pointed out, the herbicidal effect of 2,4-D is probably not due to a mere depletion of food reserves but is largely the result of other physiological disturbances.

### 1:3.2 Photosynthesis

Auxin herbicides have been found to reduce the rate of photosynthesis. Wedding et al. (1954) reported that the degree of inhibition caused by 2,4-D was related to the concentration of undissociated 2,4-D molecules in the treatment solution.

Respiration was also inhibited at high concentrations but was stimulated by small amounts of the undissociated acid molecules. Freeland (1950) observed a reduction in photosynthesis in plants treated with 30 and 100 ppm of 2,4-D, the reduction being greater with the higher concentration after 24 hours. Respiration also decreased at both 2,4-D levels but then partially or fully recovered 48 hours after treatment.

A concentration of 4000 ppm of 2,4-D decreased the uptake of carbon dioxide by Pinus resinosa seedlings. This inhibition was believed to be a direct effect on the photosynthetic process and paralleled the development of chlorosis in the needles (Sasaki and Kozlowski 1967). Carbon dioxide uptake was also inhibited in mustard plants sprayed with 2,4-D (Penner and Ashton 1966). The extent of inhibition was related to herbicide concentration and to light quality, 500 ppm of 2,4-D showing the greatest effect on CO<sub>2</sub> uptake in red and pink light than in any other light quality examined. Williams and Dunna (1961) noted that 2,4-D was most effective in reducing chlorophyll content in red light in mustard plants. The associated growth repression appeared to result from a lower chlorophyll content and consequently a lowered photosynthetic efficiency. However, in susceptible field bindweed plants sprayed with picloram, the chlorophyll content was significantly greater than in controls (Young and Feltner 1968), while in resistant *Kochia* plants the opposite was true. Penner and Ashton (1966) noted in their literature review that 2,4-D reduced the rate of photosynthesis, and the chlorophyll content in monocotyledons and dicotyledons; however, it was observed that since the photosynthetic rate was reduced first, it was not primarily caused by chlorophyll depletion.

Loustalot and Muzik (1953) suggested that injury of the mesophyll cells in velvet bean leaves sprayed with 0.001-0.01%

2,4-D was closely associated with the rapid decrease and cessation of photosynthesis. The decrease in the rate of photosynthesis occurred within 5 hours after spraying and the first visible damage to the mesophyll was within 24 hours. However even without any observed tissue damage, a concentration of 0.01% 2,4-D still caused a gradual reduction in photosynthesis over several days.

The inhibition of the photosynthetic process in the plant is apparently not the primary site of the herbicidal action of 2,4-D. Robertson and Kirkwood (1970) suggest that, unlike the direct inhibition of photosynthesis induced by triazine and phenylurea herbicides, reduction in the rate of photosynthesis by 2,4-D seems often to be an indirect effect resulting from its actions on other metabolic systems.

### 1:3.3 Oxidative-Phosphorylation Responses

The major objective of the degradation of carbon substrates by a plant is the production of energy for growth and development (Conn and Stumpf 1967). The energy available in glucose is largely released through the tricarboxylic acid (TCA) cycle when pyruvate is oxidized to carbon dioxide and water. Oxidation of the intermediates of the TCA cycle is closely related to phosphorylation, a process involving the incorporation of inorganic phosphate with adenosine-5-diphosphate (ADP) to form the energy-rich compound adenosine-5-triphosphate (ATP). Wedding and Black (1962) stated that herbicidal concentrations of 2,4-D appeared to inhibit oxidation and decrease phosphorylation at different locations in the TCA cycle. Carbon substrates of this cycle such as malate, citrate and succinate were affected by the uncoupling of phosphorylation associated with their oxidation. Wedding and Black (1962) believed this uncoupling effect by 2,4-D was large enough to account for the acute toxic responses

by plants to 2,4-D. Picloram applied at concentrations from  $10^{-3}$  to  $10^{-4}M$  has also been reported to inhibit oxidation of TCA cycle substrates (Moreland 1967). The uncoupling action by both an auxin and an auxin antagonist (two forms of 2-(2,4-dichlorophenoxy) propionic acid) was noted by Hilton *et al.* (1963) which suggested that while the mode of action of the auxin herbicides may partially be due to their effect on oxidative-phosphorylation their major lethal action is carried out through another mechanism.

#### 1:3.4 Protein and Amino Acid Synthesis

Key *et al.* (1966) suggested the herbicidal action of 2,4-D in hypocotyl cells of soybean seedlings was associated with the renewal of ribonucleic acid (RNA) and protein synthesis. This increased protein synthesis was considered to lead to massive tissue proliferation, disorganized growth and finally death of the plant. Other workers have also reported effects of auxin herbicides on protein synthesis (Sell *et al.* 1949, Fults and Payne 1956, Mann *et al.* 1965, Moreland *et al.* 1969) but there is little consistency in the results observed.

In a number of instances, 2,4-D treatments have caused an increase in protein and free amino acid contents in the stem accompanied by a decrease in these substances in leaves and roots (Wort 1964). Treatment of red kidney bean plants with 1000 ppm 2,4-D doubled the amount of protein in the stems (Sell *et al.* 1949) and altered the free amino acid content. The concentration of lysine, valine, methionine and phenylalanine increased whereas that of arginine and aspartic acid decreased. The protein content of both stems and roots of buckwheat plants sprayed with 2,4-D concentrations between 100 and 1000 ppm increased but was unchanged in the leaves (Wort 1964). Fults and Payne (1956) found 2,4-D to have variable effects on the protein and amino acid contents of different

species. Treatment with 1000 ppm 2,4-D increased the total free amino acids in the tops of sugar beet and potato plants but decreased them in bean tops. Total protein content increased in potato tops, decreased in bean tops, and remained unchanged in shoots of sugarbeet.

Mann et al. (1965) observed a moderate inhibition of the incorporation of leucine into protein in plants treated with 2 ppm 2,4-D. Muzik and Lawrence (1959) found that the spraying of bean plants (Phaseolus vulgaris L.) with 2,4-D reduced both protein and amino acid contents in the roots. However similar reductions occurred in plants which were simply uprooted and left to die. They concluded that the effect of 2,4-D on protein metabolism was only of secondary importance.

Young and Feltner (1968) examined the difference between susceptible bindweed and Kochia plants sprayed with 3 lbs/ac of picloram. The major difference observed after twenty-four hours was an increase in amino acid content in treated bindweed and a decreased amount in Kochia plants following treatment.

### 1:3.5 Enzyme Systems

Shifts in reserve food and amino acid levels in plants treated with auxin herbicides may be the manifestation of subtle changes in various enzyme systems. Many attempts have been made to determine the enzyme or enzymes affected by 2,4-D. The literature indicates that this herbicide influences a great many metabolic processes and hence involves a large number of enzymes. Crafts (1953) mentions several enzymes found to be affected by 2,4-D. Phosphatase activity is increased following 2,4-D treatment and fluctuations in levels of catalase, peroxidase and amyloplastic enzymes have been found in plant tissues, depending on length of treatment and the time at which the response was measured. Lipase, the

catalyst for lipid metabolism, decreased in activity after 2,4-D application to plants. Van Overbeek (1952) noted increased pectin methylesterase activity in auxin-treated tissues. This enzyme is associated with the breakdown of pectic substances in cell walls and its increased activity may reduce the tensile strength of young primary walls. A decrease in ascorbic acid oxidase activity in leaves of cucumber seedlings was associated with auxin-induced growth (Key 1962). The level of ascorbic acid decreased in leaves but accumulated in stems of plants sprayed with auxin concentrations of 2,4-D. Key (1962) suggested the growth promotion of the stem by 2,4-D was related to this increase in ascorbic acid, while growth inhibition in the leaves was associated with a decrease in ascorbic acid and an increase in dehydroascorbic acid. Both picloram and 2,4-D inhibited  $\alpha$ -amylase production possibly due to blockage of either RNA or protein synthesis (Moreland *et al.* 1969).

A large number of enzyme systems may be affected by applications of 2,4-D and picloram and may cause profound changes within the plant. Due to the complex interrelations of many metabolic processes, an effect on even a single enzyme could begin a chain reaction that could have far-reaching effects on plant growth and development. Crafts (1961) notes that 2,4-D may bring about a stabilization of an enzyme level that would normally be rising or falling. Such an effect could cause a change in plant response but may have little relation to the lethal effects of 2,4-D.

### 1:3.6 Nucleic Acid Metabolism

All the metabolic reactions mentioned previously require enzymes whose formation is DNA-directed in the nuclei, chloroplasts and mitochondria. It is obvious that continued growth, development, and general maintenance of life depend upon active DNA synthesis in the plant. DNA is essential for the synthesis of ribonucleic acid (RNA) and proteins.

Crafts (1961) and Key et al. (1966) state that the basic biological role of auxin is ultimately connected with nucleic acid metabolism. Changes in this metabolic process can be related to the onset of cell division and proliferation. Knypl (1965) working with indole-3-acetic acid (IAA) found that the regulation of protein synthesis by this auxin was mediated through its influences on RNA metabolism. Low concentrations of IAA enhanced the rate of elongation of pea internodes and this was accompanied by an increase in DNA-dependent RNA synthesis and a subsequent increase in protein level. Moreland (1967) observed that the regulation of RNA and protein synthesis has been ascribed not only to endogenous growth regulators such as IAA but to auxin herbicides as well.

West et al. (1960) reported a net increase in RNA and protein content of mature cucumber tissue 5 days after spraying plants with 400 ppm of 2,4-D. In 2,4-D-treated soybean plants, the level of RNA was double the amount in control tissues 48 hours after a 500 ppm application (Key and Hanson 1961). A 2,4-D concentration of  $10^{-3}M$  prevented the loss of RNA from cotton cotyledon tissue during culture either by stimulating RNA synthesis or by inhibiting its degradation (Basler and Nakazawa 1961). Key (1963) found that during a 12 hour incubation period, 500 to 800 ppm of 2,4-D inhibited the loss of RNA in corn mesocotyl tissue. Apparently this was not due to increased RNA synthesis but to an inhibition of the activity of ribonuclease (RNA'ase). Growth of the tissue was also inhibited in this concentration range. The degradation of RNA however was promoted by 10 ppm of 2,4-D by enhancing the metabolic breakdown of pre-existing and newly synthesized RNA. Chrispeels and Hanson (1962) postulated that the herbicidal action of 2,4-D lies in a renewal of nuclear activity in the tissue, leading to an abnormal

synthesis of RNA and protein, and uncontrolled tissue proliferation. They suggested that the cytochemical basis of auxin herbicide action lies in a reversion of tissues to a meristematic state. Soybean hypocotyls from 2,4-D-sprayed plants contained increased total RNA contents compared with controls. The increase was largely in ribosomal-RNA and to a lesser extent, soluble-RNA. Soluble- or transfer-RNA acts as a specific carrier of individual amino acids and is found soluble in the cytoplasm; ribosomal-RNA is a component of ribosomes, the site of protein synthesis (Cohen 1965). High levels of 2,4-D were observed by Fites et al. (1969) to stimulate synthesis of both ribosomal-RNA and DNA in soybean hypocotyls. Messenger-RNA, to carry genetic information from DNA to the ribosomes, was also produced. As a result of this stimulated nucleic acid synthesis, active tissue proliferation was initiated in the stem. Key and Shannon (1964) also found that growth-promoting concentrations of 2,4-D induced a net synthesis of ribosomal-RNA as measured by the incorporation of C<sup>14</sup>-nucleotide by soybean hypocotyls. They concluded that low concentrations of 2,4-D enhanced the growth rate by stimulating RNA synthesis. Herbicidal levels of 2,4-D were also found to increase RNA synthesis. The increase in RNA was directly proportional to increasing concentrations of 2,4-D from 110 to 2200 ug/ml. Smaller increases in DNA and protein were also found. Cells accumulated more ribosomal-RNA than soluble-RNA. Key and Shannon (1964) observed that this increase in RNA was directly coincident with massive tissue proliferation and lateral root formation.

Several reports in the literature describe an effect on RNA'ase activity by auxin herbicides. Shannon et al. (1964) observed an inhibition of enzyme activity in excised corn mesocotyl sections treated with high 2,4-D concentrations. However, applications of 2,4-D to intact corn plants promoted



or inhibited growth depending on the concentration employed but protein and nucleic acid contents, as well as RNA'ase activity, increased with increasing concentrations of 2,4-D even into the herbicidal range (Shannon et al. 1964). They proposed that excessive nucleic acid and protein synthesis would preclude normal cell development and function, and hence might serve as the biochemical basis of 2,4-D herbicidal action.

Picloram activity on nucleic acid levels resembles that of 2,4-D (Malhotra and Hanson 1966, 1970). Sensitive cucumber and soybean plants responded to 1000 ppm of picloram by increasing RNA and DNA content 24 hours after application whereas the RNA level of resistant grasses (barley and wheat) changed very little. Herbicidal concentrations of picloram induced a decrease in net DNA content of the resistant species. Ribosomal- and soluble-RNA decreased in the resistant species and increased in the sensitive plants. It was thought that the presence of higher levels of native bound nucleases (RNA'ase and DNA'ase) in resistant plants prevented the accumulation of nucleic acids. Picloram reduced the level of bound nucleases in both sensitive and resistant plants but the net concentration was still greater in the resistant species. Malhotra and Hanson (1966) concluded that auxin herbicides such as picloram and 2,4-D increase the formation of a DNA-fraction of nucleic acids, probably associated with the nucleolus. This is followed by excessive ribosomal-RNA synthesis leading to aberrant growth and eventually death. Liang et al. (1969) support this hypothesis of altered nucleic acid metabolism giving rise to unregulated growth. 2,4-D applied to grain sorghum (Sorghum vulgare Pers.) caused a variety of chromosome abnormalities, increased the chromosome number and also cell size as a result of nucleic acid alteration. Chrispeels and Hanson (1962) observed that, due directly to the effect of 2,4-D on nucleic acid metabolism, the nuclei of affected cells became swollen and formed large prominent nucleoli.

#### 1:4 Purpose of the Present Study

There is interest in Australian forestry in the development of safe and effective selective herbicides. The Tordon herbicides, Tordon 22K (potassium salt of picolinic acid) and Tordon 50D (1 part triisopropanolamine salt of picloram and 4 parts 2,4-D), have proved highly toxic to eucalypt vegetation in pine stands (Bachelard et al, 1965). Moreover, they have been shown to have markedly different effects on the growth and development of pine seedlings (Bachelard and Boughton 1967). Using 0.2% commercial formulations made up in water, the concentration being designed to simulate the dosage pine seedlings would probably receive when young plantations are treated with low volume applications of herbicides, Bachelard and Boughton (1967) found Tordon 50D to be highly toxic to actively growing pine seedlings and Tordon 22K to have relatively little effect. Tordon 50D caused significant reductions in shoot growth and general seedling health, and occasionally killed the shoot spines. Although Tordon 22K had no effect on shoot growth, it caused some morphological changes. Certain seedlings had yellowed and flattened needles near the tip and swollen apices with newly developed small scale-like needles. In some instances, lateral branching was stimulated near the apex. Obviously this selectivity of two commercial formulations of the Tordon herbicides on pine species is of extreme interest and practical significance in the development of safe and effective herbicide treatments for field use.

The variety of investigations reviewed in this chapter show that although auxin herbicides are known to affect a wide range of metabolic systems, their primary action is still not unequivocally defined. The differential effects of the two commercially available Tordon herbicides on pine seedlings seems to offer a system in which to investigate the primary

cause(s) of action of these herbicides, and permit a better general understanding of herbicide selectivity. To examine the many possible causes of this differential effect, it will be necessary to reduce the number of experimental variables to as few as possible. If a primary mode of action can be established using the two commercial herbicides, then the results will be examined more closely using the pure chemicals, picloram and 2,4-D.

## CHAPTER 2

### PRELIMINARY STUDIES OF HERBICIDE ACTIVITY

The discussion in Chapter 1 on the problems associated with herbicide selectivity suggests that the differential toxicity of Tordon 50D and Tordon 22K to P. radiata seedlings may be caused by numerous factors. An initial investigation of several of these factors is reported in this chapter.

The differential toxicity of Tordon 50D and Tordon 22K may be characteristic of a particular herbicide concentration or may be due to the 2,4-D component of Tordon 50D as suggested by Bachelard and Boughton (1967). Differences in uptake, translocation and/or breakdown of the two herbicides within the plant may also possibly account for their differential action. Furthermore, picloram is present as the triisopropanolamine salt in Tordon 50D but as the potassium salt in Tordon 22K.

It was hoped that a preliminary study would indicate not only the significant factors involved in the differential toxicity but also suggest lines for further investigation.

#### Experiment 1

Effects of concentration of pure and commercial herbicide formulations on P. radiata seed germination and early seedling growth.

P. radiata seeds were soaked in distilled water for 24 hours, surface dried and 20 seeds plated out on filter paper in each of two petri plates for each treatment. Initially, 10 mls. of herbicide solution were added to each plate, further amounts of the appropriate solution being added to keep the filter paper moist throughout the germination period.

Solutions used were Tordon 22K and Tordon 50D, made up

in water from commercial formulations supplied by the Dow Chemical Co. and applied at rates of 1, 10, 25, 50 and 100 ppm. Tordon 22K contains the potassium salt of picloram (4-amino-3,5,6-picolinic acid) and Tordon 50D contains the triisopropanolamine salts of picloram and 2,4-D in a ratio of 1:4. Hence, in addition, the pure chemicals, 2,4-D and picloram were dissolved in 0.005M triethanolamine and applied in the same concentrations as for Tordon 22K and Tordon 50D with the exception that 2,4-D was applied also at 200 and 400 ppm. Controls were run using distilled water and 0.005M triethanolamine.

The seeds were allowed to germinate in darkness at a temperature of 20°C for 15 days.

### Results

The germination of P. radiata seeds as indicated by splitting of the seed coat and protrusion of the radicle was not significantly affected by the Tordon 22K and picloram concentrations used (table 2.1). 2,4-D at concentrations of 100 ppm and greater lowered germination percent compared to controls. The lowest germination (55%) of seeds in contact with 2,4-D occurred at the highest concentration of the chemical. Tordon 50D at concentrations of 25 ppm and lower had no effect on final germination achieved but germination was markedly reduced by higher concentrations. Concentrations of 50 and 100 ppm Tordon 50D produced final germination results significantly lower than those found with the highest concentration of 2,4-D.

Table 2.1 Final germination percent of P. radiata as affected by herbicidal treatment after 15 days

TREATMENT	<u>CONCENTRATION (PPM)</u>							
	0	10	10	25	50	100	200	400
Controls-water only	82.5	-	-	-	-	-	-	-
Controls-0.005M tri.	87.5	-	-	-	-	-	-	-
Tordon 22K	-	90.0	95.0	82.5	82.5	80.0	-	-
Tordon 50D	-	80.0	82.5	82.5	37.5	15.0	-	-
Picloram	-	85.0	90.0	82.5	80.0	82.5	-	-
2,4-D	-	72.5	82.5	85.0	85.0	72.5	67.5	55.0

Although germination percent was unaffected by the concentrations of Tordon 22K used, subsequent seedling development was inhibited (Fig. 2.1 (a)). Seedlings developed swollen and curled hypocotyls and elongation of both roots and hypocotyls was greatly reduced at herbicide concentrations of 10 ppm and above. Seedlings treated with picloram concentrations 10 ppm and greater showed malformations resembling those treated with Tordon 22K (Fig. 2.1 (b)). The cotyledons of seeds in treatments with both chemicals appeared weakened, somewhat curled and at lower herbicide concentrations, seemed to force their way out of the seed coats earlier than in control seedlings.

All concentrations of Tordon 50D markedly affected seedling development (Fig. 2.1 (c)). Cotyledons failed to emerge from the seed coats in many instances and seedlings had short, thick hypocotyls. Root development was drastically reduced compared with that of control seedlings. Seedling

development was most inhibited at the highest concentrations which, as already noted, also affected seed germination.

Concentrations of 2,4-D 10 ppm and higher affected seedling development in a similar manner to that shown with Tordon 50D (Fig. 2.1 (d)). Hypocotyls and root elongation was markedly reduced and hypocotyls were swollen. No bending or curling of the hypocotyl-root portion, as was found in seedlings treated with Tordon 22K and picloram, occurred in the 2,4-D or Tordon 50D treatments. Tordon 50D at 50 and 100 ppm (containing 2,4-D concentrations of 200 and 400 ppm respectively) reduced root elongation and development more markedly than treatments with pure 2,4-D at concentrations of 200 and 400 ppm. These results indicate that the 2,4-D content of Tordon 50D at the concentrations used is not solely responsible for the toxicity of the herbicide to P. radiata seeds.

#### Experiment 2

Effects of the triisopropanolamine and potassium salts of picloram on P. radiata and eucalyptus seedlings

Two solutions of picloram were prepared, one containing the potassium salt (0.2% picloram in 0.05M KOH) and the other the triisopropanolamine salt (0.2% picloram in 0.05M triisopropanolamine). Each solution contained 0.7% (v/v) Decol T/70 as a surfactant. Using a micropipette, a 20  $\mu$ l droplet of solution was applied to the shoot tips of one-year old, potted P. radiata seedlings. Five seedlings of uniform height and development were used for each treatment and the plants were kept in a glasshouse under day-lengths of 16 hours (natural daylight supplemented by fluorescent and incandescent lights). Plants were watered regularly and examined daily for herbicide affects over a four week period.

In addition, a comparison was made of uptake, translocation and breakdown of both salts when applied to

P. radiata seedlings (Bachelard, unpublished). Picloram ( $^{14}\text{C}$  carboxyl labelled) was made up at 0.2% in 0.05M KOH and in 0.05M triisopropanolamine. Again five seedlings were used for each treatment; three seedlings were prepared for autoradiography and two seedlings for extraction purposes seven days after treatment. Acidified acetone extracts of macerated pine tissue were taken up in 0.1 ml methanol and a chromatogram run using Whatman no. 1 paper and n-butanol:triethanolamine: water (5:1:2, v/v) as solvent.

The uptake, translocation, and breakdown of both salts when applied to E. viminalis and E. polyanthemous seedlings were also examined by the same techniques (Bachelard, unpublished) Results.

There were few morphological effects by either the potassium or the triisopropanolamine salt to P. radiata seedlings at the concentration used (Fig. 2.2). The bases of needles near the shoot tip were yellowed in some cases but this was not confined to a particular salt treatment. All seedlings had green and actively growing shoot tips throughout the experiment.

Autoradiographs of treated seedlings showed similar patterns of herbicide uptake and translocation with both salts (Fig. 2.3 (a) and (b)). Labelled material was concentrated in the upper needles and around the growing tip. The autoradiograph of the chromatogram of the extracts from labelled seedlings showed all the radioactivity to be present as picloram (Fig. 2.4).

The results of the similar study using eucalypt seedlings showed no consistent differences in uptake with the picloram salt used (Fig. 2.4). Variations occurred according to species treated, surfactant used, and plant portion examined. Shoots contained greater amounts of labelled picloram



than roots and there was slightly greater uptake of potassium (K) salt than of triisopropanolamine (P) salt in shoots using the surfactant Decol T/70. Chromatographed extracts of shoots and roots showed the radioactivity present as picloram and not as any breakdown product of the two salts (Bachelard, unpublished).

These results indicate that the radioactively labelled potassium or triisopropanolamine salts are not differentially broken down over a seven day period in either P. radiata or eucalypt seedlings. While no measurement of quantitative distribution is available, the results suggest a similar qualitative uptake and distribution pattern. Hence, the difference in type of salt is unlikely to explain the differential toxicities of Tordon 50D and Tordon 22K.

### Experiment 3

Effects of concentration of pure and commercial herbicide formulations on P. radiata and eucalyptus seedlings.

Herbicide solutions of Tordon 22K and Tordon 50D from commercial formulations were made up in water containing 0.7% Decol T/70 surfactant and applied at concentrations of 0.05, 0.2, 1.0 and 4.0%. Pure 2,4-D and picloram were dissolved in 0.05M triethanolamine at concentrations of 0.05, 0.2 and 1.0% and dissolved in 0.2M triethanolamine at 4.0% and surfactant added. The following herbicide combinations plus surfactant were also employed; 0.05% picloram + 0.2% 2,4-D, 0.2% picloram + 0.8% 2,4-D and 1.0% picloram + 4.0% 2,4-D.

A 20 $\mu$ l droplet of herbicide solution was applied to the shoot tip of one-year-old P. radiata seedlings. Five seedlings per treatment were used.

One leaf of the fourth leaf pair from the tip of one-year-old E. viminalis seedlings was also treated with herbicide solution. Using lanolin paste, a plastic tube (1 cm dia. x 1.5 cm length) to contain the herbicide solution

in a uniform area was fixed to the leaf surface close to the mid-rib of the leaf. A 20 $\mu$ l droplet was applied to the leaf surface through the plastic tube using a micropipette. Again five seedlings were used for each treatment.

Control treatments were carried out with both P. radiata and E. viminalis seedlings using 0.05M and 0.1M triethanolamine solutions containing 0.7% Decol T/70. Plants were kept in a glasshouse under conditions described in Experiment 2. A daily examination of all seedlings for herbicide effects was made over a four week period. The leaves of each E. viminalis seedling were counted and recorded prior to treatment, and a record was kept of percent of leaves killed in each treatment over the experimental period.

### Results

The response by P. radiata seedlings varied with the herbicide and concentration used. There was little effect on shoot tips by Tordon 22K at 0.05 and 0.2% concentration (Fig. 2.6 (a)) but at 4.0%, growing tips were browned and needle bases of some surrounding needles were yellowed after four weeks. In extreme cases of injury by 4.0% Tordon 22K, the immediate tip was killed and the stem below the tip excessively swollen (Fig. 2.6 (b)). The effects of picloram were similar to those caused by Tordon 22K; at the highest concentration, tips were killed and surrounding needles browned (Fig. 2.7 (a)) and lower concentrations caused a slight chlorosis of needles about the tip (Fig. 2.7 (b)).

All growing tips were killed by Tordon 50D at concentrations 0.2% and greater (Fig. 2.8). Needles at the tip were browned and dead, and at 1.0% concentration, some apices were swollen and adventitious buds had formed. The lowest concentration caused needle bases to yellow and browned the upper portions of needles about the tip. Shoot tips of two seedlings of the five treated were killed by 0.05%

Tordon 50D.

2,4-D treatment resulted in greater injury to P. radiata shoot tips than Tordon 50D at a comparable concentration (Fig. 2.9). At concentrations of 1.0 and 4.0%, 2,4-D killed shoot tips and surrounding needles and some stem apices were swollen and adventitious buds had formed. Lower 2,4-D concentrations caused less injury. Tips were killed in some cases and needles browned but there was no apical swelling or tissue proliferation even when plants were examined two months after treatment.

A mixture of 1.0% picloram + 4.0% 2,4-D killed tips and upper needles of all seedlings treated (Fig. 2.10 (a)). Slight yellowing of needle bases and browning of their upper portions occurred with the mixture 0.2% picloram + 0.8% 2,4-D but the majority of shoot tips were unaffected (Fig. 2.10 (b), right). Less plant injury resulted with this mixture than with either 0.2% Tordon 50D or 2,4-D at 0.2 and 1.0% concentrations. Slight chlorosis of shoot needles of some plants occurred with 0.05% picloram + 0.2% 2,4-D (Fig. 2.10 (b) left).

Triethanolamine was not toxic to P. radiata seedlings when used at 0.2M concentration (Fig. 2.11). The response by plants to herbicides dissolved in 0.2M triethanolamine appears to be due solely to the herbicide content of the solution.

Toxicity of herbicides to eucalypt seedlings was measured in percent of leaves killed (Fig. 2.12). With Tordon 22K, the earliest symptom of toxicity was leaf discolouration above and below the treated leaf. One week after treatment with 1.0 and 4.0% Tordon 22K, tips were dead and most leaves had changed from dark green to a brownish-red colour. After four weeks plants, irrespective of concentrations of Tordon 22K used, carried only dead leaves on dry, brittle stems.

Tordon 50D was not as effective at all concentrations used as Tordon 22K in killing eucalypt seedlings. There was no general leaf discolouration and toxicity was gradual and progressed over the four week treatment period. Shoot tips were killed one week after treatment with 4.0% Tordon 50D and by the fourth week, 100% leaf kill was achieved at this concentration. The lowest concentration (0.05%) gave 51% leaf kill and all tips were alive after four weeks.

Tordon 50D at 4.0% and all Tordon 22K concentrations resulted in higher percentages of leaves killed than picloram under the conditions of this experiment. 81% of leaves were killed using 0.2 and 4.0% picloram. Leaves were discoloured on some plants after one week, and tips were killed following the 1.0 and 4.0% treatments. Tips remained alive four weeks after treating plants with 0.05% picloram although 43% of leaves were killed.

2,4-D was the least effective herbicide in killing eucalypt leaves and plants. One week after treatment, most leaves on all plants were green and healthy with only the treated leaf browned and curled. Shoot tips remained alive in all but the 4.0% 2,4-D-treated plants. By the end of the treatment period, 40% leaf kill was obtained with the 4.0% concentration and only 23% with the 1.0% 2,4-D-treatment.

The herbicide mixture 1.0% picloram + 4.0% 2,4-D resulted in 81% of leaves killed and not surprisingly, was the most effective of the mixtures used. It resulted in a higher percent of leaves killed than either 1.0% picloram or 4.0% 2,4-D used alone but less than the result obtained with 1.0% Tordon 22K. The toxicity of the other two herbicide combinations was variable and they appeared less effective (but not significantly) in killing eucalypt seedlings than the mixture using the highest herbicide concentrations.

The high percent of leaves killed by all concentrations of Tordon 22K used is difficult to explain, especially when compared with results using similar concentrations of pure picloram. A similar discrepancy is apparent when damage caused by certain percent concentrations of picloram in Tordon 50D is compared with that caused by the same concentrations in Tordon 22K. It is very unlikely that the 2,4-D content of Tordon 50D would have a suppressing effect on the toxicity of picloram to eucalypts. Nor can the effectiveness of the Tordon 22K concentrations used here be adequately explained by variations in environmental or plant factors. The most reasonable explanation for the high effectiveness and uniformity of the results of Tordon 22K is in the quality of the particular commercial herbicide batch from which the concentrations were made. The similarities in results for pure picloram and Tordon 50D at comparable concentrations is important and suggest that the toxicity of Tordon 50D to eucalypts depends essentially on its picloram and not its 2,4-D constituent.

Eucalypt seedlings were unaffected by 0.05M or 0.2M triethanolamine solutions. However, on some plants, the treated leaf was browned and mottled, particularly at the higher concentration.

### Conclusions

The results of Experiment 1 indicate that the effect of Tordon 22K on the seed germination and seedling development of P. radiata is essentially the same as that of pure picloram at the same concentration. By contrast, the effects of Tordon 50D do not correspond either quantitatively (seed germination) or qualitatively (seedling development) to that of 2,4-D alone at the same concentrations. This suggests that the effects of Tordon 50D on P. radiata seed germination and

subsequent development result from the synergistic action of picloram and 2,4-D.

There was no difference in morphological response of P. radiata seedlings to 0.2% picloram applied as the potassium salt or as the triisopropanolamine salt. Autoradiographs of labelled seedlings also showed qualitatively similar absorption and translocation of both salts. Autoradiographs of seedling extracts gave no evidence of breakdown of either picloram salt within the pine seedling. The results of treating the eucalypt seedlings with the same salt formulations showed essentially similar action by both picloram salts. The basis of the differential action of Tordon 22K and Tordon 50D is hence unlikely to result from the different picloram salt formulations found in the commercial herbicides.

The study of various herbicide concentrations has shown Tordon 22K to be highly effective in killing eucalypt seedlings and to have relatively little effect on pines at concentrations 1.0% and lower. Tordon 50D was toxic to pine seedlings at all but the lowest concentration used and was not as effective as Tordon 22K in killing eucalypts. 2,4-D was more toxic to pines than to eucalypts and in this respect, resembled the toxicity pattern of Tordon 50D. However, again the effectiveness of Tordon 50D was not wholly explained by its 2,4-D content, nor does an additive effect of picloram and 2,4-D appear to explain completely Tordon 50D toxicity.

The basis of the differential effect of Tordon 22K and Tordon 50D would seem to lie outside the factors investigated in this preliminary study and might be found in biochemical differences of selectivity to these herbicides.

Field trials have shown that picloram was highly effective in killing eucalypt coppice growth when applied as a high volume spray at a concentration of 0.04% (Bachelard et al.

1965). It was further recommended that low volume applications using 0.2% picloram would be just as effective and economical in killing unwanted eucalypt vegetation. The work in this chapter shows that this rate of picloram treatment had relatively little effect on P. radiata seedlings when applied as Tordon 22K but killed shoot tips and upper needles when Tordon 50D was used. Both 0.2% Tordon 22K and Tordon 50D would effectively kill eucalypt seedlings. Furthermore, Bachelard and Johnson (1969) found that concentrations of 0.2 lbs/ac (25 ppm) and 0.5 lbs/ac of commercial Tordon 50D applied to the soil surface inhibited the subsequent development but not the emergence of P. radiata seedlings.

The concentrations chosen were ones which could be expected in normal forestry practise (Bachelard and Johnson 1969).

In view of the findings in this chapter and of those reported in the literature (Bachelard et al. 1965, Bachelard and Boughton 1967, Bachelard and Johnson 1969), it was decided to examine tissues of P. radiata treated with 0.2% concentrations of the commercial herbicides Tordon 22K and Tordon 50D. The concentration was chosen because picloram, when used on an operational scale in pine and eucalypt forests, is applied at concentrations of around 0.2 - 0.5 lbs/ac (10 gal. of 0.2 - 0.5% solution /ac). The results presented in this chapter indicate that the differences in behaviour of the commercial formulations are largely but not entirely due to the known pure chemical constituents. For this reason and so that results could have practical significance to field studies, the commercial herbicide formulas were used in much of the following work which examined the basis of the differential response. Once a possible primary mode of action was established, pure 2,4-D and picloram was tested to determine whether or not they were the responsible agents.

In the following chapters the results are reported of a broad scale anatomical and histochemical investigation, undertaken in the hope that an evaluation of the effects obtained would lead to a better understanding of the biochemistry and physiology of Tordon herbicide action and also to indicate where more detailed research would prove most profitable.



## CHAPTER 3

ANATOMY OF GERMINATING SEEDS

In the initial evaluation of plant responses to Tordon herbicides, metabolically active, and highly susceptible tissues were examined. Germinating seeds incorporate a large number of physiological processes which may be affected by herbicides since the embryo itself is composed primarily of young meristematic tissues, and the normal cell processes of elongation, division and differentiation occur extensively as it germinates and develops. Bachelard and Johnson (1969) reported that the early seedling development of Pinus radiata is markedly affected by both Tordon 22K and Tordon 50D, so in the initial stages of this study the effects of herbicide treatment on germinating seeds were examined.

3:1 Materials and Methods

Pinus radiata seeds were soaked in distilled water for 24 hours, surface dried, and plated out on filter paper in petri plates. This initial soaking enabled the seeds to develop an intercellular environment essential to the functioning of enzymes and hence, to become metabolically operative again (Stone 1957, Mayer and Poljakoff-Mayber 1963), before exposing them to the herbicides. It also provided a more valid initial test of the requirement for stratification of the seeds used.

To each petri plate were added 10 mls of one of three solutions; distilled water as a control; 25 ppm picloram supplied as Tordon 22K, and 25 ppm picloram plus 100 ppm 2,4-D supplied as Tordon 50D. The herbicides were made up in water from commercial formulations. The seeds were germinated in darkness at 21°C, and drops of the appropriate solutions were

added as required throughout the period of germination to keep the filter paper moist. Samples of seed material were removed 24 hours after the initial plating-out and at 48 hour intervals thereafter up to eleven days. The seeds were fixed in 3% glutaraldehyde in 0.025M phosphate buffer, dehydrated and embedded in polyester wax (Steedman 1957, Sidman et al. 1961). Sections were cut 20  $\mu$  thick on a Cryo-Cut Cryostat microtome Model 849C at 8°C, and mounted on glass slides coated with 0.1% gelatin. After removal of wax with ethanol, the sections were stained with 0.05% toluidine blue according to Feder and O'Brien (1968).

Tissues were examined under a light microscope and photographed with a Nikon F 35 mm camera. Cell lengths were determined from measurements of 100 cells taken from six seeds from each treatment.

### 3:2 Results

#### 3:2.1 Germination and Gross Morphology

Stratification of the seeds used in this study was unnecessary as the germination of unstratified seeds and those stratified at 4°C for four weeks was virtually the same in all treatments (Table 3.1).

Table 3.1

Germination percent and germinative energy index (G.E.I.) of P. radiata seeds as affected by pre-germination and germination treatment (each mean determined from 100 seeds).

Pre-germination treatment	Germination Treatment		
	Water	Tordon 22K	Tordon 50D
1. Unstratified seeds - soaked 24 hours in water	80% 0.416 (G.E.I.)	80% 0.457	85% 0.441
2. Stratified seeds - soaked 24 hours in water, surface dried, plated out after 4 weeks at 0°C	75% 0.461	100% 0.453	80% 0.323

The resumption of embryo growth as indicated by the splitting of the seed coat and protrusion of the radicle was similar in all treatments. Radicle emergence began on the fifth day of treatment and by day 7 germination was complete. Under the conditions of this experiment there was no significant effect of herbicide treatment on the percent of seeds germinating or their germinative energy index (G.E.I.) (Table 3.1). Bartlett's index of germinative energy as described by Grose (1963) was used here.

However, the subsequent seedling development was greatly affected by herbicides. Fig. 3.1 indicates diagrammatically, the morphological differences in growth form after 15 days germination in the various treatments. The most marked response was the suppression of root elongation in both herbicide treatments, and the thickening of the hypocotyl. Tordon 50D-treated seeds developed short, swollen hypocotyls with drastically reduced roots. The hypocotyls of seeds germinated in Tordon 22K were distinctly thicker than those of the controls, and the roots of these seedlings were severely checked in length, became swollen, and were curled

in several cases. Results presented in Table 3.2 indicate the differences in length of the hypocotyls and radicles of treated seedlings compared with those of the controls. Measurements indicate the marked effect of both the Tordon herbicides on root elongation. Hypocotyls of herbicide-treated seedlings were thicker than those of the controls and elongation was significantly reduced by Tordon 50D.

Table 3.2

Measurement (in cms) of germinating seeds 15 days after plating-out (population size in brackets).

Treatment	Hypocotyl	Radicle
Tordon 50D (20)	$0.5 \pm 0.03$	$0.3 \pm 0.02$
Tordon 22K (15)	$0.9 \pm 0.10$	$0.4 \pm 0.04$
Controls (20)	$0.8 \pm 0.06$	$3.9 \pm 0.33$

The cotyledons in both herbicide treatments appeared severely weakened and flaccid, with the terminal portions remaining inside the seed coats for some time. Tordon 50D seedlings had shrivelled and suppressed cotyledons, while those in Tordon 22K were more variably affected, being reduced in some cases and extremely elongated, twisted and curled in others. In all treatments, the cotyledons were chlorophyllous, but no chlorophyll extractions were carried out to determine what effect if any, the herbicide treatments had on chlorophyll content.

### 3:2.2 Measurements of Cell Lengths

The suppressed elongation of both the hypocotyl and root zones of Tordon 50D-treated embryos and the root tissues of those treated with Tordon 22K indicates an effect on either cell division, cell elongation or both. The effects on

cell elongation were examined by measuring cells in each of three regions of the embryo as shown in Fig. 3.2.

Photographs taken of tissues from different treatments were enlarged and measurements of cells were made from the photographs.

Information on cell division in this study, is qualitative and confined to remarks on occurrence and polarity of division. Quantitative data requires sections to be cut at a fixed depth in embryos, in a level plane, not always possible in this study, and the use of staining techniques other than that employed here, to bring out the various aspects of the mitotic cycle. Furthermore, Romberger (1963) notes that apart from problems in determining frequency of cell division there is also a general lack of information on duration of cell division. Hence, recording the number of dividing nuclei in a tissue region may not give reliable information on frequency of division.

(i) Pith Cells

The pith cells below the shoot apex in the developing seedling generally stain lighter than the cells of the surrounding tissues due to a relatively large volume of vacuolar material in their cytoplasm. They are more irregular in size and shape compared to the cells of the cotyledons and hypocotyls, and are not rigidly aligned in columns. Cell division in the pith cells is normally in a transverse plane across the long axis of the embryo. Cell measurements show an overall decrease in cell length over the 11-day germination period irrespective of treatment (Fig. 3.3) due to cell division at intervals during germination and early seedling growth. During the first three days, there was no significant effect of treatment on cell lengths. Lengths initially increased prior to cell division because of swelling due to water inhibition, but by day 5, the onset of cell division in tissues receiving Tordon 50D significantly reduced cell length measurements. The smaller pith cells in

these tissues (Fig. 3.4 (e)) can be compared with those of control sections (Fig. 3.4 (a)). After 11 days the values of cell lengths for the Tordon 50D-treated seeds were somewhat less than those of the controls. The curve for Tordon 22K-treated cells lagged behind that of Tordon 50D, but showed a similar pattern in that cell division also appeared to be stimulated.

Cell division had already commenced in Tordon 22K-treated tissues by day 5 (Fig. 3.4) resulting in slightly smaller cells than those found in the controls. This difference was accentuated by day 7. Numerous divisions were apparent in the control tissues by day 7, and the difference in cell length measurements between the controls and Tordon 22K-treated tissues at this time could be due in part to a reduced expansion of the divided cells in the herbicide treatment. By day 11, the shortest cells were found in Tordon 22K-treated tissues; the longest in the controls.

Comparison of both the herbicide-treated tissues and the controls on the 11th day of treatment showed two types of cell in the area under examination; large cells found in the central pith, and smaller cells containing large round nuclei situated below the shoot apex. Measurements of the lengths of both types of cell were included in the values for day 11.

In the small-cell area below the apex in both herbicide treatments, there were many more actively dividing cells compared to the controls. An apparent disruption in the normal, ordered arrangement of cell division has also occurred. In the controls (Fig. 3.5), almost all cells in the area under examination divided in a plane parallel to the long axis of the embryo, i.e. in a transverse plane which leads to shoot extension growth. However, in the herbicide-treated tissues (Figs. 3.6 and 3.7) some cells were dividing

in oblique and longitudinal (across the long axis of the embryo) planes, suggesting that the normal regulation of the plane of division may be disrupted by herbicide treatment. This would lead to the reduced extension growth and the increased tissue volume observed in the developing seedlings receiving herbicide.

Additionally, the herbicides caused a marked increase in the vacuolation of the subapical cells. This is especially obvious in the tissues receiving Tordon 50D (Fig. 3.7) where there is considerable disruption and tearing of the cell walls, and an apparent breakdown in cytoplasmic integrity.

#### (ii) Cortical Cells

The same general pattern of diminishing cell lengths over the germination period was found for the cortical cells of the upper hypocotyl as for the pith cells (Fig. 3.8). Cell lengths in all treatments were unaltered up to day 3 but by day 5, Tordon 22K treatment caused a significant reduction in cell length.

Cell length measurements were further reduced after 7 days of treatment, partly due to numerous cell divisions occurring and partly because of the failure of cells to expand. Considerable vacuolation of these cortical cells had taken place (Fig. 3.9 (c)) but the subsequent expansion of these cells gave an increase in cell lengths by day 11.

Cell lengths were not significantly altered by Tordon 50D treatment for five days but by 7, cell lengths in tissues receiving this treatment corresponded to those of the Tordon 22K treatment. Significant cell elongation in Tordon 50D-treated tissues occurred only after day 9 giving an increase in cell lengths by day 11. Some cell division and an increase in vacuolation of cortical cells was evident in Tordon 50D-treated tissues by days 7 and 9 (Fig. 3.9 (e) and (f)) and both sections showed some possible reduction in cell elongation.

In the controls, cell lengths remained constant for five days, after which increasing numbers of cell divisions resulted in a progressive reduction in average cell length. Vacuolation of control cells by day 7 was markedly less than in the herbicide-treated tissues (Fig. 3.9 (a)) but was actively occurring by days 9 and 11 (Figs. 3.9 and 3.10).

The control cortical cells were still actively dividing and expanding after 11 days (Fig. 3.10) whereas the herbicide-treated cells appeared to have reached a more advanced and final stage of development. The cells receiving Tordon 22K were highly vacuolated, had a thin parietal layer of cytoplasm, and nuclei were closely adpressed to the cell wall (Fig. 3.11). The Tordon 50D-treated tissues were also highly vacuolated but, as with the pith cells discussed earlier, there appeared to be some breakdown of cell walls, and disruption of cytoplasm and nuclei (Fig. 3.12). In some instances cells lacked cytoplasm and nuclear content.

### (iii) Cotyledon Cells

The cotyledons of seeds treated with Tordon 50D were in most instances shrivelled and weakened, and their elongation was greatly suppressed. Tordon 22K-treated cotyledons were more variable, either being greatly reduced in length or extremely elongated and curled. Microscopic examination of the cotyledon cells at different stages of germination showed the same general pattern of decreasing cell length with time (Fig. 3.12), as in the pith and cortical cells but, at the final measurement after 11 days of treatment, there was no significant difference in the cell lengths of control and herbicide-treated tissues (Table 3.3).

At five days after treatment, the cotyledon cells in the Tordon 50D-treated seeds were significantly longer than in the controls or in the Tordon 22K-treated seeds due,



apparently, to delayed cell division (Fig. 3.14 (e)). No cell divisions were apparent in the cotyledons prior to those observed at five days; cell division occurred only in the control and the Tordon 22K-treated seeds (Fig. 3.14). At nine days, the cotyledonary cells of the Tordon 50D-treated seeds were significantly shorter and those of the Tordon 22K-treatment significantly longer than the controls. At this stage, the cotyledonary cells in the control, and the Tordon 50D-treated cells were actively dividing. The differences in cell length appear due to a slower rate of cell expansion following division in the Tordon 50D-treated tissues (Fig. 3.15 (e) and (f)). Cells in the Tordon 22K treatment were of two distinct types. The first of these is comparable to those of the control tissues (Fig. 3.15 (c)). The second occurred in extremely elongated and curled cotyledons. In these tissues, while cell division is apparently considerably reduced, elongation is seemingly unaffected or somehow enhanced, resulting in larger than average cells (Fig. 3.15 (d)).

By day 11, cell length measurements were similar in all treatments but the stage of development of the cells was affected markedly by herbicide treatment. The control cells were obviously still actively dividing and expanding with, in some instances, evidence of vacuolation (Fig. 3.16). In the herbicide-treated tissues (Fig. 3.17 and 3.18), cell division appeared complete and the cells were almost completely vacuolated. As with the pith and hypocotyl cells described earlier, many cells were devoid of cytoplasmic and nuclear material and in some areas there was some rupturing and breakdown of cell walls.

On day 11, further measurements of cell length using six different seeds from each treatment were taken. The wide variation in values for the cortical cells in the upper hypocotyl is shown in Table 3.3. All but one of the

mean values for Tordon 22K-treated cells are significantly longer than the controls. In the range of values for cotyledon cells, the great variation in cell length between cotyledons of different seeds, irrespective of treatment, is obvious. Because of this variation, there are no significant differences in the final mean values for cotyledon cell length due to herbicide treatment.

Table 3.3

Average cell lengths (in microns) of the cortical cells in the upper hypocotyl 11 days after treatment. (Each mean based on measurement of 100 cells).

Seed No.	Controls	Tordon 22K	Tordon 50D
1	25.0 $\pm$ 0.67	39.4 $\pm$ 1.18	25.9 $\pm$ 0.66
2	25.6 $\pm$ 0.65	28.3 $\pm$ 0.64	18.6 $\pm$ 0.49
3	26.2 $\pm$ 0.80	31.1 $\pm$ 0.86	21.1 $\pm$ 0.58
4	26.4 $\pm$ 0.84	29.7 $\pm$ 0.76	27.8 $\pm$ 0.80
5	20.4 $\pm$ 0.63	25.1 $\pm$ 0.70	31.0 $\pm$ 0.74
6	24.8 $\pm$ 0.63	42.7 $\pm$ 1.10	30.3 $\pm$ 0.78
$\bar{X}_6$	24.7 $\pm$ 0.70	32.7 $\pm$ 0.87	25.8 $\pm$ 0.67

Table 3.3

Average cell lengths (in microns) of cells in the basal portion of the cotyledons 11 days after treatment. (Each mean based on measurement of 100 cells).

Seed No.	Controls	Tordon 22K	Tordon 50D
1	18.3 $\pm$ 0.55	22.4 $\pm$ 0.60	23.3 $\pm$ 0.63
2	24.0 $\pm$ 0.76	25.1 $\pm$ 0.65	19.9 $\pm$ 0.58
3	21.8 $\pm$ 0.58	19.9 $\pm$ 0.56	17.5 $\pm$ 0.59
4	18.2 $\pm$ 0.50	23.3 $\pm$ 0.61	34.7 $\pm$ 1.06
5	17.7 $\pm$ 0.57	24.4 $\pm$ 0.42	18.4 $\pm$ 0.69
6	29.2 $\pm$ 0.68	No measurement	19.4 $\pm$ 0.54
$\bar{x}_6$	21.6 $\pm$ 0.60	23.0 $\pm$ 0.57	22.2 $\pm$ 0.68

### 3:2.3 Shoot Apical Meristem

The activity of the cells of the shoot apical meristem causes an increase in length and volume of the epicotyl during germination. The epicotyl in Pinus radiata seeds is conical in form and quite symmetrical (Fig. 3.19) and consists of meristematic tissues; the promeristem and the ground meristem. The promeristem possesses the apical initials which are characterized by relatively large, lightly-stained nuclei (Fig. 3.19 (b)). The initials differ very little from their recently formed daughter cells and hence it is difficult to determine their exact number and limit (Eames and McDaniels 1947). The cells of the ground meristem have deeply-stained and somewhat smaller nuclei. The promeristematic region occurs to a depth of 2-3 cells in the upper portion of the epicotyl and to some extent along its flanks. The ground meristem lies just above the central pith and there is no distinct line of demarcation between the

two regions.

In gymnosperm apices the control of orientation of the plane of cell division is much less rigid than in angiosperms (Romberger 1963) and increase in cell number of the promeristem is accomplished by both transverse and longitudinal divisions and occasional division in an oblique plane. In the inner ground meristem, cell division is primarily in a transverse direction resulting in columns of cells parallel to the long axis of the embryo. Similar findings have been reported for Pinus strobus L. (Spurr 1949) and for P. lambertiana Dougl and P. ponderosa Laws (Sacher 1954).

In this study, herbicide treatment had no apparent effect on the developing shoot apex by day 9 (Fig. 3.20). Small cells with large dark-stained nuclei and dense cytoplasm developed on the flanks of the apical dome and by day 9, primordial buds were initiated in several embryos irrespective of treatment. Apices were generally conical in shape, and had cells with large nuclei and lightly stained cytoplasm.

By day 11, the herbicide treatments had an obvious effect on the growth and continued development of the shoot apices. While apices of the control embryos were conical and had increased in volume over the germination period (cf. Fig. 3.19 and Fig. 3.21), those of embryos treated with Tordon 22K and Tordon 50D appeared flattened and reduced in size, suggesting possible reduction in individual cell expansion. Primordial buds were well developed in all embryos and in some cases, secondary primordial buds were forming.

Although cell division in gymnosperm apices can occur in any plane, there appears to be a significantly greater number of irregularities in the plane of division of herbicide-treated tissues compared with control sections. Division in

the ground meristem of control embryos was predominantly in a transverse plane and resulted in an overall increase in apical height. Examination of apices of Tordon 22K-treated embryos (Fig. 3.22) and of those treated with Tordon 50D (Fig. 3.23) suggests that cell division in both the pro- and ground meristems occurred frequently in oblique and longitudinal directions as well as in transverse planes. The orientation of divisions coupled with apparent reduction in cell expansion could account for the seemingly broader and flatter apices of herbicide-treated embryos compared with those of the controls.

#### 3:2.4 The Radicle and Root Meristem

Since the root apex is lacking in developing appendages such as cotyledons and primordia, it is generally regarded as being simpler in gross structure, and its development and growth as less complicated than the shoot apex (Eames and McDaniels 1947). Romberger (1963) notes that the lack of nodes and internodes in roots results in more uniform growth and less variation in size and shape of the apex compared with those of developing shoots.

The root apex is composed of ground meristem tissue terminated by the apical initials, the number of which may vary at any particular time. The extent of the initials is difficult to assess but the cells in contrast to those of the ground meristem are relatively lightly-stained and possess large, lightly-stained nuclei. Hence, in this regard, the root initials are comparable with those of the shoot apex. The root meristem initials are typically deep-seated and are separated from the external environment by the root cap. The root cap consists of a central region, the column, and surrounding area, the peripheral tissue. The column is characteristic of gymnosperm roots (Spurr 1949) and is composed

of cells in long files. This tiered construction is maintained by the predominance of divisions in a transverse plane. In the peripheral tissue, cells are obliquely orientated and cell division is frequently across this plane.

The onset of germination of the seed, as evidenced by radicle protrusion, is accomplished by cell elongation in the lower hypocotyl area, and by an increase in cell number in this and in the root meristem regions. In actively growing embryos, the area of rapid cell division is characterized by small, compactly-arranged cells, almost completely filled with dense cytoplasm and large nuclei. Behind this meristematic tissue are located the regions of elongation, cell differentiation and maturation. These areas are not easily delineated due to different rates of differentiation by different cells at different distances behind the apex. The rate of elongation will also affect the location of various zones. In slowly growing roots, where the process of cell elongation is suppressed, differentiating tissues will extend closer to the root apex than would normally occur in rapidly growing roots. Hence, root tissue in which growth is severely altered or checked would have a poorly developed region of elongation, a zone of cell differentiation extending almost to the root apex, and a meristematic region somewhat reduced in size.

The root apices of P. radiata embryos were more sensitive to Tordon herbicide treatment than the shoot apices. The direct effect of herbicides on the root tissues was to check their elongation and enhance lateral expansion and swelling, resulting in short, thickened roots. Roots thus affected would obviously have their normal functions impaired. This would have profound significance on normal development of the plant.

Examination of sections of root apices under the

light microscope showed that herbicides had had no apparent effect on development after three days of treatment (Fig. 3.24). However with the beginning of cell division and elongation in the lower embryo regions between days 5 and 7, the influence of herbicide treatment on development and growth became more obvious.

After five to seven days of germination, seed coats split and radicles emerged from the majority of seeds irrespective of treatment. In root tissues of the control treatment, cell divisions in the meristematic zone produced numerous cells immediately behind the apex and on its flanks beneath the root cap. Further behind the apical meristem, cell elongation, particularly in the cortex, produced large, thin-walled cells containing small nuclei (Fig. 3.25). Cells of the vascular tissue in this region were elongated and narrow.

Pronounced contrasts to normal root development were found in tissues treated with the Tordon herbicides. After five days, cell division and elongation had commenced in the radicles of Tordon 22K-treated seeds. However, the elongation of cells was markedly reduced compared to those in control tissues and by day 7, the herbicide-treated roots were comparatively short, swollen and curled in some instances (Fig. 3.26 (a)). Unequal rates of elongation of the cortical cells on either side of the vascular cylinder caused the curling of the root tips while a general reduction in individual cell elongation accounted for the reduced root length compared with controls. An increase in root thickness was due to two factors. Firstly, groups of cortical cells in the region of elongation had expanded laterally causing large air spaces within the tissues. The affected cells in some instances had broken walls and there

appeared to be some crushing of adjoining cells (Fig. 3.26 (b) and (c)). Secondly, the increased thickness of the cortex was also due to a lateral increase in the number of cells. These cells were small and had dense cytoplasm with large nuclei (Fig. 3.26 (a) and (b)).

Cell elongation was severely checked in roots treated with Tordon 50D. Roots were thicker and growth in length reduced compared with those of the control or Tordon 22K treatments. Groups of cortical cells were slightly vacuolated and swollen in some instances causing the crushing of adjoining cells. Their length was markedly reduced (Fig. 3.27 (a)). More significantly, an increase in the number of cells adjacent to the vascular cylinder resulted in a layer of proliferating cells (Fig. 3.27 (b) and (c)). This increase resulted apparently from changes in the plane of cell division from predominantly transverse to longitudinal (Fig. 3.27 (c)). These cells had large, round nuclei and in some areas of the root tissue rows of cells had ruptured apart. The origin of these proliferating cells is not clear, but the tissues developed result both from a failure of the newly-formed cells to elongate and an increase in the number of cells dividing in a transverse plane.

After 11 days of germination, the effects of herbicide treatment on root tissues were marked. Roots of the control seeds continued to elongate and were long, slender and well developed (Fig. 3.28). The cortex comprised a narrower layer than in tissues receiving herbicide, and the cells were typically elongated and thin-walled. Xylem cells were formed in the vascular tissue. There was no evidence in any of the control root tissues examined of abnormal cell proliferation or irregular swelling of cortical cells.



Massive cell proliferation about the vascular cylinder was evident in root tissues treated with each of the herbicides. Root tissues of Tordon 22K-treated seeds were swollen due to a sheath of rapidly and abnormally dividing cells and to swelling of random groups of cortical cells (Fig. 3.29). Tordon 50D-treated tissues were similar to those treated with Tordon 22K (Fig. 3.30). There was evidence of altered planes of cell division in root tissues of both treatments. However, the elongation of cortical cells in Tordon 50D-treated roots appeared even more suppressed and the zone of cell proliferation further developed than in roots treated with Tordon 22K.

Areas of broken tissue were found in root sections of many of the herbicide-treated seeds examined. These may have been caused by internal pressures developed by the proliferating tissue or by random swelling of some cortical cells or possibly by a direct effect of the herbicides on cellular integrity.

### 3:3 Discussion

The concentrations of the herbicides Tordon 22K and Tordon 50D used here had no effect on the germination percentage of P. radiata seeds but markedly affected the morphology of the root, hypocotyl and cotyledons of developing seedlings. The overall growth of the root and hypocotyl was severely checked resulting in the shortening and swelling of these organs. Cotyledons were reduced in length in most seedlings and were often twisted and flaccid. Similar findings have been reported for the effects of herbicides on seedlings of Pinus resinosa Ait. (Kozlowski and Sasaki 1968a, 1968b, Sasaki and Kozlowski 1968a) and for P. radiata (Johnson 1967). Root growth of P. resinosa seeds was checked by atrazine, monuron and 2,4-D

when seeds were maintained in direct contact with the herbicides (Sasaki and Kozlowski 1968 (a)). In particular, 2,4-D at 100 ppm showed a strong depression of root growth and plants had swollen stems with shrivelled or sometimes elongated cotyledons. Kozlowski and Sasaki (1968b) further reported that concentrations of 2,4-D as low as 0.001 ppm reduced root elongation and caused morphogenic changes, although shoot growth was stimulated at concentrations as high as 1.0 ppm. They also demonstrated that shoot growth was affected less than was root development by picloram at the same concentration. Root elongation was inhibited at concentrations of 0.01 ppm and greater although shoot development was not significantly altered up to 1.0 ppm. The development and extension of roots of P. radiata seeds germinated in soil treated with 0.2 and 0.5 lbs/ac picloram or 2.0 and 5.0 lbs/ac 2,4,5-T was severely limited (Johnson 1967). Four months after treatment, seedlings grown in soil treated with picloram had fewer fine roots and more weakly developed root systems compared with those of either control seedlings or those of seedlings grown in soil treated with 2,4,5-T.

Concentrations of the herbicides used in this study had greater effect on the root than on the shoot portion of the seedlings. There were, however, profound alterations in the development and growth of cells of the hypocotyl and cotyledons.

The pith and cortical cells of hypocotyls of P. radiata embryos were affected similarly by both Tordon herbicide treatments. Cell elongation was reduced in the pith cells of herbicide-treated tissues and, although photographs of comparable regions of cells of the upper cortex from all treatments suggests a reduced elongation of cortical cells in herbicide-treated tissues, qualitative

results (Fig. 3.9, Table 3.3) indicate that after 11 days of treatment cortical cell elongation, especially in Tordon 22K-treated tissues was in fact enhanced. It is possible that these cells grew larger but more probable that cell division rate was reduced. Cell elongation of the cotyledonary cells was more variably affected over the germination period, but by day 11, measurements of cell length in these organs of six seeds per treatment were not significantly different. The effect of the herbicides on suppressing cell elongation is more conclusively demonstrated in root tissues. In some seeds treated with Tordon 22K, unequal rates of cell elongation or depressed elongation on one or other side of the root resulted in root curvature. By contrast, in Tordon 50D-treated roots elongation of cells was uniformly suppressed. The growth of the control roots was due to cell elongation and to cell division in a transverse plane. Herbicide-treated root tissues were reduced in length primarily because of checked cell elongation. This agrees with the observation of Rojas-Garciduenas and Kommedahl (1958) on elongation of cells of soybean radicles treated with 2,4-D. They suggested that reduced root growth following 2,4-D treatment was due more to a reduction in individual cell elongation rather than a decreased rate of cell division.

Although the rate and frequency of cell division were not determined in this study, the normal plane of cell division may have been affected by herbicide treatment. This was less obvious in the shoot portion of the plant than in the root tissues. Subapical cells of herbicide-treated seeds divided primarily in a transverse plane. However, there were frequent instances of divisions occurring in oblique and longitudinal directions. Also by comparison with the

control treatment, small-diameter cells were more numerous in herbicide-treated tissues immediately below the shoot tip. These cells showed reduced expansion and some irregularity in division. No record was made of the number of abnormal cell divisions in the shoot apex because of differences in the plane of the sections and the few replicates available for study.

However, modification of the pattern of cell division was readily observed in root tissues. Cells flanking the vascular cylinder divided at right angles to the normal plane of division and, without further cell expansion, continued to divide, creating a sheath of meristematic cells. The subsequent proliferation of this layer exerted pressure on cells external to it and resulted in large air spaces in the cortex. The number of rows of cells in the cortex also increased and this, in addition to the mass of proliferating cells caused the observed thickening of the root tissue.

Other workers have found similar tissue proliferation in roots of seedlings treated with 2,4-D and picloram. Some of the earliest reports were by Eames (1949, 1950) who observed the proliferation of a layer of cells in bean roots treated with 2,4-D, resulting in the swelling of the roots and the compression and rupture of cells outside the proliferation zone. This sheath of rapidly dividing cells arose from abnormalities in the plane of cell division. A similar ring of proliferating tissue surrounding the outer periphery of the stele was found in roots of 2,4-D-treated corn seedlings (Hoshaw and Guard 1951). The treatment of non-resistant bindweed seedlings with 2,4-D stimulated abnormal cell division in the roots resulting in a zone of proliferating cells that caused the swelling of the root tips and the subsequent fragmentation and destruction of the cortex (Whitworth and Muzik 1967). Picloram has also been reported to induce abnormalities in cell division in root tissues. A meristematic zone arose from changes in the

plane of division of cells in roots of Phaseolus vulgaris seedlings treated with 72 µg/plant of Tordon 22K (Fisher et al. 1968). The continued development of this proliferating tissue eventually caused the rupture of some cortical cells and stretching of the epidermis. An increase in cell division with abnormalities in plane of division has also been reported in leaf tissue treated with Tordon 22K (Fisher et al. 1968, Scifres and McCarty 1968).

The cellular integrity of tissues of P. radiata treated with the Tordon herbicides was disrupted. This is an important observation in the light of work subsequently presented. In particular, the cells of the upper hypocotyl and the pith demonstrate precocious vacuolation and maturation leading to an early cessation of their division and elongation. Both Tordon 22K and Tordon 50D appeared to cause disruption of the cell wall and cytoplasmic integrity of many of the cells. The breakdown of the plasmalemma and cellular content became obvious as treatment continued. Cells of the cotyledons similarly showed effects of herbicide treatment on vacuolation and cellular integrity. Kozlowski and Clausen (1966) found cellular integrity of the needles of Pinus resinosa and Picea glauca (Moench) Voss to be disrupted by the herbicide 3-amino, 1,2,4-triazole. The shape of the mesophyll cells was altered and their vacuolation was greatly increased.

The results presented in this chapter are supported in part by a recent publication of Kozlowski and his co-workers (Wu et al. 1971). Using concentrations of 50 and 100 ppm of 2,4-D and picloram supplied in direct contact with germinating Pinus resinosa seeds, they found the effects of both picloram and 2,4-D to be similar but picloram appeared more toxic at comparable dosages. The symptoms of herbicide treatment first appeared in root tissues and after six days

of treatment, both elongation and division of cells in these tissues had ceased. After eighteen days of treatment, numerous small cells with large nuclei and dense cytoplasm proliferated freely outside the vascular core resulting in abnormal thickening of the stem. Cell division of this layer when examined on cross-section, appeared to be irregular and in randomly orientated planes. Outer cortical cells were swollen and collapsed, and had ceased to divide and elongate.

In this chapter, no explanation for the cessation of cell elongation or for the proliferation of some cells has been put forward. Wu et al. (1971) suggest that the stimulation by herbicides of cell division in the inner cortical region may merely be related to the fact that in many plants this region remains meristematic longer than the outer region and therefore it is probably easier to induce continuation of cell division in this area. Weintraub (1953) suggests further that where normal tissue growth depends on polarized cell division, the addition of compounds such as 2,4-D and picloram may provide competition for endogenous growth regulators disturbing the regular plane of cell division and resulting in aberrant cell proliferation and tissue malformations.

Root-tip thickening of P. radiata seedlings appears to result primarily from a stimulus to activity of meristematic cells, leading to extensive cell proliferation. Chrispeels and Hanson (1962) suggest that the aberrant growth following herbicide treatment is similar to the growth associated with meristematic tissue. This was based on the observation of a high nuclear activity and RNA synthesis in proliferating tissue of soybean hypocotyls treated with 2,4-D.

The effect of Tordon 22K and Tordon 50D on RNA and other cell constituents of P. radiata seedlings will be

examined in the following chapter.

### Conclusions

There are three main effects of the Tordon herbicides on P. radiata tissues emphasized in this study:

1. A marked reduction in individual cell elongation resulting in greatly reduced root growth;
2. An effect on cell division, particularly of cells outside the vascular cylinder, giving rise to a proliferating layer of small, aberrantly dividing cells resulting in abnormal thickening of the roots;
3. A disruption of cellular integrity, possibly related to the plasmalemma, causing the early vacuolation and maturation of parenchyma cells.

## CHAPTER 4

MICROSCOPIC HISTOCHEMISTRY OF GERMINATING SEEDS

As pointed out in the Introduction, auxin herbicides may affect many plant processes making it extremely difficult to distinguish between primary and secondary effects. It seemed reasonable to launch a broad-scale investigation of the effects of herbicides on some major plant constituents with a view to determining the extent and the rate at which these are influenced by herbicides. The results of such a study could indicate where more intensive research should be concentrated.

For this initial survey, it was decided to use histochemical techniques. Such techniques can be highly specific for particular plant chemical constituents especially if controls are used. They consist primarily of localization and identification procedures, and give data directly in terms of cells or cell parts. The majority of techniques give qualitative and not quantitative information and are dependent upon changes in colour, in intensity, or in position within the plant to indicate changes in composition or quantity of the constituent being examined. However, small shifts in the amount or position of cell constituents may occur that are not perceivable by microscopic histochemical procedures. Nevertheless, histochemistry has proved useful in the examination of many plant processes and developments. Anatomical and developmental changes in germinating seeds of Paulownia tomentosa Steud. were studied histochemically by Rickson (1968) and similarly, Fosket and Miksche (1966) examined the growing shoot apical meristem of Pinus lambertiana Dougl. Histochemical procedures have also been used to note chemical and structural changes associated with flower induction in Chenopodium album (Gifford and Tepper 1962), in Lolium temulentum L. (Knox and Evans 1966) and in cauliflower (Sadik and Ozbun 1967). Recently



a histochemical study of the phenomenon of seed dormancy was completed (Biswas et al. 1970).

In this section, the results of histochemical tests on germinating P. radiata seeds treated with Tordon 22K and Tordon 50D are given. The major plant constituents studied were proteins (only metabolically inactive storage proteins and not enzymes), carbohydrates (insoluble polysaccharides), lipid substances, and ribonucleic acid.

#### 4:1 Materials and Methods

The preparation of material for histochemical examination was similar to that described in Chapter 3 for the anatomical studies. Unstratified seeds of Pinus radiata were soaked in distilled water for 24 hours and placed on filter paper in petri plates containing a few mls of Tordon 22K (25 ppm picloram), Tordon 50D (25 ppm picloram, 100 ppm 2,4-D) or distilled water as a control. The petri plates were kept in darkness at 21°C, except when seeds were removed at intervals of two days, beginning after one day of treatment until 11 days after treatment.

For the examination of protein and carbohydrate content, the seeds, with their seed coats removed, were fixed in 3% glutaraldehyde in 0.025M phosphate buffer for 24 hours at 4°C, dehydrated and embedded in polyester wax (Steedman 1957, Sidman et al. 1961). Sections 20 $\mu$  thick were cut on a Cryo-Cut Cryostat at 8°C and mounted on glass slides prior to staining.

During the above dehydration and embedding procedure, lipids are readily extracted from the tissues. This necessitates using fresh (unfixed) frozen sections. Seeds from which the seed coats had been removed were placed in a 10% solution of DMSO (dimethyl sulphoxide) in water at 4°C overnight, after which they were embedded in 12% gelatin medium containing 1% DMSO by melting a hole in the gelatin plate with a hot needle

and inserting the tissue into the hole.<sup>1</sup> The melted gelatin was allowed to set at 4°C before rectangular blocks containing the tissues were cut out. The blocks were then placed on a quick-freeze unit of the cryostat and frozen. Sections were cut 30µ thick at -20°C and picked up on warm glass slides. The use of DMSO aids in reducing the temperature gradient between tissues and the gelatin mixture during freezing. This prevents the formation of ice crystals in the cells and results in easier cutting and less tearing of sections.

For ribonucleic acid study, a modification of Carnoy's fixative containing acetic acid-ethanol (1:3 v/v) was used (Knox and Evans 1966). Following fixation for six hours at 4°C, the tissues were washed in running water for 24 hours, dehydrated and embedded in paraplast wax (melting point 56-57°C). Sections (5µ thick) were then cut on the cryostat at 8°C.

#### 4:1.1 Histochemical Procedures

(i) Protein - Sections were stained for two hours with a solution of 0.05% bromophenol blue in 2% acetic acid containing 1% mercuric chloride to localize total plant storage proteins (Pearse 1960). The technique is not primarily a histochemical one but is based on the binding of all proteins by bromophenol blue in the presence of mercury. The amount of dye bound is directly proportional to the amount of protein present (Mazia et al. 1953). No controls are necessary when using HgCl-BPB since this is a highly specific stain. Basic proteins (histones) or proteins high in basic amino acids were stained with 0.1% bromophenol blue in water for 10 minutes, rinsed in 0.5% acetic acid, and washed in running tap water.<sup>2</sup> Controls were

(a) hydrolysis in hot (60°C) 5% trichloroacetic acid for 3 hours

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1. Dr. B. Knox - Botany Dept. A.N.U. - personal communication

2. Dr. M. Howell - Zoology Dept. A.N.U. - personal communication

to remove nucleic acids (Knox and Evans 1966) and (b) acetylation, following hydrolysis, in a 10% solution of acetic anhydride in pyridine for 18 hours at room temperature (Jensen 1962). This step blocks the staining of lysine-rich histones.

(ii) Carbohydrates - The PAS (periodic acid-Schiff's) schedule and aldehyde blockade of Feder and O'Brien (1968) were used to detect total carbohydrates of insoluble polysaccharides. The aldehyde blockade was carried out using a saturated solution of 2,4-dinitrophenylhydrazine (DNPH) in 15% aqueous acetic acid. The basis of the PAS reaction is the oxidation of polysaccharides by periodic acid forming aldehyde groups which then combine with leucofuchsin (Schiff's reagent) to produce colour complexes. The aldehyde blockade precedes the PAS schedule and serves to cover up any naturally occurring aldehyde groups or any introduced by the glutaraldehyde fixation. The PAS schedule will stain only those aldehyde groups attached to polysaccharide chains. As a control, if, after the aldehyde blockade, the periodic acid step is left out of the PAS procedure there should be no staining of sections by Schiff's reagent.

Starch was localized in the tissues using a solution of 0.2gm of iodine crystals dissolved in 2% potassium iodide in water (Jensen 1962).

(iii) Lipids - For the localization of lipid substances, sections were stained with freshly prepared and filtered 0.1% Sudan black B in 70% ethanol (Jensen 1962). Controls were run using the pyridine extraction technique outlined by Jensen (1962) for complete lipid removal.

(iv) Ribonucleic acid - Azure B stain was used to detect RNA in tissue sections. Sections were deparaffinized with xylene, hydrated through an alcohol series, and stained with an 0.25 mg/ml solution of Azure B in citrate buffer, pH 4.0 for 2 hours at 50°C (Jensen 1962). After staining, sections were differentiated in pure TBA (tertiary butyl alcohol) for 30

minutes to remove excess stain, mounted with a coverslip in TBA, examined and photographed.

The specificity of Azure B for RNA was confirmed by following the method of Dickinson and Heslop-Harrison (1970). After staining, sections are digested with RNA'se, examined and re-photographed. Highly purified RNA'se (obtained from British Drug Houses (Aust.)) was made up at 0.1% concentration in glass-distilled water. The pH was adjusted to 6.0 with a minimum of 0.1N NaOH. The effect of pH on RNA removal was tested by treating sections with glass-distilled water at pH 6.0 (Jensen 1962).

After staining with Azure B, the sections were first washed in running tap water and allowed to air dry. Slides containing several sections were then placed on water-moistened filter paper in petri plates. A drop of RNA'se solution was placed on the tissue section photographed previously and simultaneously, another section was treated with distilled water as a control for pH. The petri plates were incubated at 37°C for 3 hours. After treatment, sections were washed well in running tap water, mounted in TBA, and re-examined. The material removed from fixed-tissue sections may be considered as exclusively RNA since the RNA'se sample was highly pure and free of any proteolytic activity. Because of its high specific enzymatic action, the use of RNA'se is preferred over other less specific extraction techniques involving perchloric acid or hot hydrochloric acid (Brachet 1953).

## 4:2 Results and Observations

### 4:2.1 Proteins

The majority of seed proteins are reserve or storage proteins and are digested enzymatically during germination to form amino acids and peptides. It has also been observed that storage proteins can be utilized in carbohydrate synthesis (Ching 1965, 1966). The breakdown (catabolism) and

utilization of storage proteins is considered essential to sustain the growth of the developing embryo.

In Pinus radiata seeds, the cells of the primary endosperm contain a large amount of storage protein. The embryo also has a large portion of the total protein content, especially in the cells of the cotyledons. Storage proteins in both the embryo and primary endosperm are spherical bodies of various sizes and stain dark blue with mercury-bromophenol blue. Tissue sections stained with bromophenol blue for basic proteins (histones) were no different in staining intensity compared with those stained for total protein content (Fig. 4.1). It was assumed therefore, that the majority, if not all, protein bodies in the primary endosperm were basic in nature. The protein bodies appear to be structurally composed of a dark, heavily-stained outer shell with an unstained central core. The exact nature of the core is unknown but may be some type of crystalloid inclusion (Altschul et al. 1966). This type of protein body is similar to the core-type described by Horner and Arnott (1965) for *Yucca* seed protein.

As expected, the protein bodies in the primary endosperm are broken down during the germination period. After seven days an appreciable change was seen in the number of protein bodies per cell in the primary endosperm of control tissues (Fig. 4.2). With a decrease in number, the protein bodies coalesced and the average size of the ones remaining increased. These fragmented into units smaller than the original protein bodies and gradually disappeared, beginning in the cells closest to the embryo and continuing out to the seed coat. It is quite possible that storage proteins of the embryo were utilized first, prior to day 7; this may account for changes in the primary endosperm being seen only after a period of seven days.

Herbicide effects on reserve protein in the primary

endosperm are difficult to assess (Fig. 4.2). Qualitative examination after 11 days shows a possible larger number of protein bodies of a smaller size in Tordon 22K-treated tissue compared with the controls. The Tordon 50D-treatment shows a large number of protein bodies per cell of apparently larger size compared with the controls. Whether these are real effects attributable to herbicide-treatment is difficult to determine. Increasing cell vacuolation and development also makes it difficult to determine the effects of the herbicides on protein synthesis and utilization in the embryo. In the cortical cells of the upper hypocotyl, storage protein bodies are very much smaller with a large central core. The cells themselves are densely packed with numerous protein bodies but after 11 days, vacuolation has forced the storage proteins against the cell walls (Fig. 4.3). During germination, these smaller protein bodies coalesce into larger particles, eventually being broken down as growth continues.

It is possible that the Tordon herbicides do not affect storage proteins. In an extensive survey by Moreland and co-workers (1969) of a wide range of herbicides, it was shown that picloram had no effect on leucine incorporation in protein synthesis in excised soybean hypocotyls. The auxin herbicide, 2,4,5-trichlorophenoxyacetic acid, did give a 67% reduction of leucine incorporation in treated tissues compared with controls. Only the gibberellin-controlled induction of  $\alpha$ -amylase formation was inhibited by picloram. Inhibition of this hydrolase enzyme could be due to a decrease in protein or RNA synthesis but since picloram was ineffective in other assays, Moreland et al. (1969) suggest that picloram was directly interfering in the gibberellin control mechanism.

Mann et al. (1965) also studied the effects on leucine incorporation by a number of herbicides. Since a higher concentration of 2,4-D (5ppm) did not inhibit leucine

incorporation at any greater rate than a lower one (2ppm) in barley and *Sesbania* seedlings, they concluded that its influence was not directly on protein metabolism but on some possible energy-yielding reactions, perhaps affecting ATP levels.

The lack of any marked effect of the herbicides on storage proteins of *P. radiata* seeds indicates that detailed investigation of the effects of herbicides on the metabolism of storage proteins would be unlikely to yield useful information on the mechanism of herbicide action.

#### 4:2.2 Carbohydrates

In the normal germination sequence, stored carbohydrates such as starch are metabolically degraded into sucrose and glucose, essential sugars for continued embryonic growth and development (Koller et al. 1962). As cells continue to divide and elongate, complex polysaccharides are built up from the products of starch metabolism and are used in cell wall construction. Starch metabolites are also utilized in respiration and in the synthesis of proteins and enzymes.

Starch grains are present in tissues in the early stages of germination. Hatano and Asakawa (1964) observed the presence of starch in both the embryo and primary endosperm of *Pinus densiflora* Sieb. & Zucc. and *Pinus thunbergii* Parl. seeds at the start of germination. As growth of the embryo continued, starch decreased from the primary endosperm. It was both synthesized and utilized in the embryo and gradually increased throughout the germination period, especially in the cells of the root cap.

Substances that are stained by the PAS treatment are entirely carbohydrate in nature and include the insoluble cell wall polysaccharides and starch grains. Lipoidal materials are not stained, having been extracted during the fixation

and dehydration schedule. Water soluble sugars are not localized because of aqueous solutions and the washings that are required in the PAS procedure. Cytoplasm also remains colourless but nuclei may stain a light pink.

P. radiata seed material was examined by the PAS technique. No changes in cell wall polysaccharides due to the presence of herbicides during germination could be detected. Subtle changes do obviously take place as the cells grow and their walls increase in thickness and stability, but changes in relative proportions of constituents of the walls cannot be detected by the PAS technique. It cannot be used as a differential cell wall stain (Jensen 1962). Any changes in the cellulose component go unobserved; cellulose generally being unstained by the PAS technique (Feder and O'Brien 1968).

It was observed in the preceding chapter that the massive tissue proliferation, cell wall rupturing and general swelling of the cortical cells of root tissue was most apparent after 11 days of herbicide treatment. Root tissue was examined therefore after 11 days to determine what changes, if any, these anatomical alterations had on starch synthesis and metabolism. The IKI procedure for starch localization was used here. It is a brief and relatively simple technique, entirely reproducible, and is based on the well known iodophillic properties of starch. The length of the starch molecule determines the final colour of the reaction products; newly synthesized starch of short molecular length will stain red to violet, the longer the molecular chain, the bluer and darker the colour of the starch grain becomes (Jensen 1962). This latter starch will be referred to here as "storage starch" only to distinguish it from newly synthesized starch.

After 11 days, starch grains were present in the root caps of the embryos irrespective of treatment. There was however, a greater amount of storage starch in the herbicide-



treated root cap column and peripheral cells compared to the controls (Fig. 4.4). Many of the starch grains in the control tissue appeared newly synthesized. The cells of the root apical meristems of both herbicide and control tissues were free of starch bodies.

It was pointed out previously that the roots of germinating seeds in both herbicide treatments were greatly reduced in length and abnormally swollen. This was due partly to massive lateral cell proliferation above the root apex and outside the vascular core. Cortical cells outside this sheath of proliferating cells in Tordon 22K-treated tissues contained a large amount of newly synthesized and storage starch (Fig. 4.5). The high starch content of these cells continued as far as the meristematic region of the root. Pith cells were relatively free of any starch, but occasionally some cells of the proliferating tissue contained recently synthesized starch.

Similarly, cortical cells in Tordon 50D-treated tissues contained a large amount of storage starch, and to a lesser extent, newly synthesized starch (Fig. 4.6). There appeared to be relatively more starch per cell than in Tordon 22K-treated sections. Large, lightly stained starch grains were extensively located in the cells of the proliferation zone and were presumably synthesized here. Newly formed starch was also localized in the pith cells.

Control tissues, apart from the root caps, were free of storage and newly synthesized starch (Fig. 4.7).

It would seem, at least in the Tordon 50D-treated tissues, that storage starch was not being broken down and had accumulated in the cortical cells. At the same time, if the assumption that the small reddish starch grains are newly formed starch is accurate, then starch synthesis was also proceeding at a fast rate in these tissues.

In the Tordon 22K-treated tissues, starch catabolism was again checked. Starch synthesis occurred but the number of newly synthesized starch grains was much less than in the Tordon 50D-treated tissues. The process was either occurring at a slower rate, or newly formed starch grains were more rapidly utilized.

Porter (1962) observed in his literature review on carbohydrate synthesis that carbon, normally incorporated into cellulose and proteins in actively growing cells, will accumulate as starch or sugars if growth is inhibited. As an example, he stated that the herbicide, maleic hydrazide, inhibited the growth of sprayed wheat leaves and the starch content was found to increase rapidly in these tissues.

The inhibition of starch catabolism in the herbicide-treated tissues is undoubtedly due to the overall decrease in cell division and elongation compared with the controls where these processes rapidly utilize the products of starch breakdown.

The effect of the Tordon herbicides on carbohydrate content in P. radiata root tissues is in contrast to reports where 2,4-D has been found to deplete starch and sugar contents (Rasmussen 1947, Smith et al. 1947, Wolf et al. 1950, Wort 1951, Wort 1964). These reports however, deal with older and more developed seedlings than the young embryos employed here, with different rates and methods of application, and with, in some instances, leaf or stem tissues and not root tissues.

The decrease in demand for polysaccharide building units in root tissues of P. radiata where growth and cell division are obviously inhibited by herbicide treatment, could well be expected to result in the accumulation of starch.

#### 4:2.3 Lipids

Plant oils and fats are a major reserve of high energy food used in the growth and development of the embryo. These lipoidal substances are a heterogenous group of chemicals,

found in the cells of both embryo and primary endosperm. Their main function is for food storage but they are also found in structural components such as cell membranes.

During germination, lipids are broken down by hydrolysis with lipid enzymes, lipases. The degradation of fats into carbohydrates via the glyoxylic acid cycle gives rise to a general increase in sugars and starches in the tissues (Stumpf and Bradbeer 1959). Ching (1966) demonstrated that lipids are the major food reserve in both the embryo and primary endosperm of Douglas fir seeds and, during germination, they are metabolized to produce carbohydrates, structural components and soluble compounds in the seedling. Lipid substances were found to disappear from the primary endosperm first, being converted to sugars which were then transported to the embryo.

Lipid substances are insoluble in water but disperse readily in fat solvents such as pyridine and alcohols. Sudan black B is an excellent stain for lipids, being highly specific, but, as it is usually made up in a 70% alcohol solution, small amounts of lipids may be removed from the sections even when extreme caution is taken during staining. Further difficulties in lipid localization arise from the use of frozen tissues. Cells may be easily torn and ruptured by the freezing and sectioning procedure, and with large tissues such as whole pine seeds, sections between 20 and 30 microns in thickness must often be cut. The diffuseness and mobility of the lipids, and the thickness of the tissue sections make qualitative observations of lipid changes extremely difficult.

Lipid substances in P. radiata seeds are located throughout the embryo and primary endosperm. Seeds examined after 24 hours' treatment contained large, irregular-shaped lipid globules scattered widely throughout the tissue. These globules broke down into smaller ones as germination proceeded and were mainly concentrated in the upper hypocotyl and the cotyledons of the embryo. After nine days much of the lipid

material had disappeared from the primary endosperm although the embryo contained large amounts. Fig. 4.8 shows the upper hypocotyl region in embryos from all three treatments. The photographs of herbicide-treated tissues were taken just below the epicotyl, primarily for clarity. Most of the lipid substances are located in the pith and vascular tissues with some small amounts found in the cortex. Large quantities of lipids were also located in the cotyledons of the herbicide-treated tissues but the poor quality of these sections prevented clear photographs from being taken. There appears to be a greater concentration of lipid substances in herbicide-treated sections, especially in the pith and vascular regions compared with control tissues.

The lower hypocotyl and root tissue are the more actively growing regions of the embryo in the early stages of germination. Here the lipid globules are quite small and in the control tissue largely confined to the pith cells (Fig. 4.9). In the tissues treated with the herbicides, more lipid globules are present than in the controls. Massive amounts of fats and oils are contained in the cells in the area corresponding to the zone of proliferation discussed before, in the pith cells, and also in the cortex.

The greater concentration of lipids in the herbicide-treated seeds compared with that in the controls is due either to reduced utilization or increased synthesis of lipids or to a combination of these processes.

Comparison of sections over the 11 day germination period indicated that the lipids were not utilized in the herbicide-treated seeds to the same extent as in the controls. It would appear that the herbicide-induced inhibition of growth was responsible for the accumulation of lipids as well as that of starch.

The very high lipid content in the zone of cell proliferation of herbicide-treated root tissue is of interest.

The cells in this region are actively dividing and lipid accumulation may indicate active synthesis here. However, it would be premature to suggest an effect of the herbicides on lipid synthesis as cell elongation was not occurring and it is possible that the accumulation is due to lowered utilization.

Mann and Pu (1968) investigated the effect of some thirty herbicides on lipid synthesis and found that 2,4-D, 2,4,5-T, and picloram all stimulated the synthesis of fats in hypocotyls of hemp sesbania. However, this synthesis was observed at low levels of these herbicides and as the concentration was increased from 1mg/L to 20mg/L, the percentage of stimulation above the controls fell. They concluded that the stimulation of lipogenesis in these tissues was due to an overall growth stimulation at the lower herbicide concentrations.

Thus, it appears likely that the accumulation of both fats and starch in the herbicide-treated P. radiata seeds in this study was caused by a general growth inhibition and lack of utilization of the reserve foods.

#### 4:2.4 Ribonucleic Acid

The importance of the nucleic acids, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) cannot be overstated. They are the means whereby continued growth and development are possible. A shift in content, regardless of magnitude, in either or both nucleic acids outside their normal pattern, could theoretically pave the way to aberrant cell division and growth irregularities. DNA has a fundamental part in transmitting vital genetic information in newly-formed cells and acts as the template in the formation of RNA. The term RNA broadly applies to a group of three types of RNA; messenger-RNA, coded for enzyme synthesis, transfer- or soluble-RNA, the specific carrier of individual amino acids,

and ribosomal-RNA, a structural RNA found in ribosomes, part of the machinery of protein synthesis (Cohen 1965). RNA, while directly involved in enzyme and protein synthesis is also important in the process of cell division, elongation and differentiation. Masuda et al. (1966) found little RNA synthesis occurring in oat coleoptiles during cell expansion, the bulk of RNA in these tissues being formed prior to the main period of cell expansion. Key and Ingle (1968) however, observed that some RNA synthesis, presumably messenger-RNA associated with the nucleus, was essential for cell expansion to proceed. Key (1964) reported that RNA synthesis was closely associated with cell division as well as expansion.

In the previous chapter, it was shown that the Tordon herbicides had disrupted the normal development of cells of embryos in germinating P. radiata seed. The vital part played by RNA in the processes of cell division and elongation warranted an examination of the effects of the Tordon herbicides on the RNA content in germinating seeds.

There are, however, numerous problems associated with accurate identification and localization of RNA in plant tissues, and the choice of plant fixative and the duration of fixation are major difficulties. Generally, any method of fixation will induce some changes in the physical state of the nucleic acids. However, precipitant fixatives such as alcohol and acetic acid and in particular, Carnoy's fixative, a mixture of acetic acid-alcohol (1:3 v/v), have been found adequate in preserving nucleic acids essentially in their original state (Pearse 1960). Usually 4-6 hours of fixation at room temperature is recommended, followed by thorough washing of tissues in running water (Knox and Evans 1965, Moss 1967). Fixation of plant material in Carnoy's solution can also cause some plasmolysis of cells and may introduce variations in dye concentrations (Moss 1967). Limited plasmolysis

occurred in cells of P. radiata embryos examined in this study.

The use of paraplast wax rather than polyester wax enabled thinner sections to be cut and reduced the amount of structural damage in the tissues. The warming of slides over an alcohol flame in order to flatten the sections presents another problem. Moss (1967) notes that the loss of some RNA may occur with this procedure with prolonged exposure to high temperatures. In this study however, the effect of temperature is negligible since the period of warming the slides was very brief.

The use of basic dyes at an acid pH is a useful procedure in localizing nucleic acids, particularly when coupled with specific extraction techniques (Jensen 1962). Azure B is a metachromatic dye which stains RNA dark blue or purple and DNA a blue-green colour at pH 4.0 (Flax and Himes 1952). Proteins and cell walls free of lignin remain unstained. Azure B is widely used in animal histochemistry because of its metachromatic properties but has also gained acceptance in work with plant tissues (Jensen 1962). Dickinson and Heslop-Harrison (1970) examined nucleic acids in Lilium and Rickson (1968) used Azure B in studies of the germinating embryos of Paulownia tomentosa. Phosphates in the nucleic acids are apparently the sites of dye binding, and the closer association of phosphates in highly polymerized DNA than in RNA, leads to differential staining (Jensen 1962). The procedure used in this study, under the conditions specified, was relatively easy to employ and highly reproducible. However, staining artifacts may occur where tissue thickness varies, when cell contents are plasmolyzed or if DNA has been physically depolymerized.

Some problems of RNA localization are associated with RNA'se digestion. Electrolytes in solution may remove a variable amount of RNA from sections and even distilled water

has this effect at temperatures of 60°C and higher (Stowell and Zorzoli 1947). For this reason, it is customary to use RNA'se in glass-distilled water at 37°C for an incubation period of 1-3 hours depending on the fixative used. Pearse (1960) states that provided the fixative employed does not cause the loss of RNA and does not render it impervious to RNA'se action, the incubation time may be varied so that RNA removal is complete. Fixatives that alter the physical structure of the ribonucleoprotein may possibly alter its digestability by RNA'se. However, Bracket (1953) reported that RNA in tissues fixed with Carnoy's solution is easily removed by RNA'se. The binding of Azure B to RNA sites also has no effect on the specificity of RNA'se for RNA (Dickinson and Heslop-Harrison 1970).

A close examination of embryos in this study was centred on the cells of the vascular and cortical tissues below the shoot and above the root apices. Cell elongation as well as cellular integrity in these areas were shown to be affected by herbicide treatment (Chapter 3). An emphasis was placed on examining root tissues since suppressed cell elongation as well as considerable cell proliferation occurred in these tissues as a result of herbicide treatment.

Photographs were taken of all seeds from each treatment over the germination period. An examination of the cells of the lower cortex of embryos after 3 days showed similarities in RNA content irrespective of treatment (Figs. 4.10-4.12). Nuclei were darkly stained by Azure B indicating an appreciable amount of RNA. While RNA was also present in the cytoplasm heavy concentrations of protein bodies made accurate estimates difficult. RNA was readily extracted from tissues by a 3 hour digestion with RNA'se.

After 7 days of treatment, radicles had emerged from all seeds due to cell elongation and division. However,



there was no apparent effect of herbicide treatment on RNA levels of cells of the upper cortex (Figs. 4.13-4.15) or lower cortical cells (Figs. 4.16-4.18). Any possible changes were masked by large amounts of protein bodies in some cells and by the variations between embryos within treatments in rates of cell division, elongation, and vacuolation. Cell vacuolation was particularly well advanced in some herbicide-treated embryos (Fig. 4.17(b) and (c) and Fig. 4.18(b) and (c)) and prevented accurate comparisons of RNA contents of embryos from different treatments.

The RNA content of cells of the upper and middle cortical and vascular tissues of embryos was not markedly affected after 11 days of herbicide treatment (Figs. 4.19-4.24). Upper and middle cortical cells in particular, from embryos of all treatments, contained darkly stained nuclei and demonstrated an increase in cytoplasmic-RNA content compared with day 3 material (Fig. 4.10 and Fig. 4.22). Nuclei of cells of the middle vascular tissue in most embryos (Figs. 4.25-4.27) were not as heavily stained as nuclei of the cortical cells and presumably contained less RNA (cf. Fig. 4.22 and Fig. 4.25).

Although freshly prepared enzyme solution was used for each extraction, total RNA removal with a 3 hour digestion period became progressively more difficult. This may be indicative of a quantitative increase in nuclear-RNA or to some qualitative change; it occurred in many tissues after 11 days regardless of germination treatment. Total RNA removal from all cells was achieved by prolonged extraction with RNA'se for 6 hours.

This differential removal of RNA by RNA'se as well as the effect of pH on RNA removal are shown in root tissues from the control treatment (Fig. 4.28) and in the root tissues treated with Tordon 22K (Fig. 4.29) and with Tordon 50D

(Fig. 4.30). Glass-distilled water, pH 6.0, had little effect on RNA removal from the tissues. However, RNA was not completely extracted by RNA'se after a 3 hour digestion period from cells of the root cap, excluding the column, and from some cortical cells, particularly those of the outer cortex.

Root tissues of the germinating embryo were more affected by herbicide treatment than the upper portions (Chapter 3). It was expected that any herbicide-induced changes in RNA would be readily detectable in these affected tissues. However, tissues from embryos of the control and herbicide treatments are not comparable because in the latter suppressed elongation and cell proliferation produced shorter and thicker roots. In root tissues from the control treatment, cells in the region of cell elongation were large with small amounts of cytoplasm and had nuclei relatively low in RNA content (Figs. 4.31-4.32). Cells immediately above the root apex were smaller and contained large darkly-stained nuclei and large amounts of cytoplasmic-RNA (Figs. 4.33-4.34). In tissues from embryos treated with herbicide (Figs. 4.34-4.40), cells above the root apex were similar to those of the controls (cf. Figs. 4.28, 4.29 and 4.30). This area was more extensive in herbicide-treated tissues than in control tissues and because of this, they appeared to contain a greater amount of cytoplasmic-RNA. Vacuolation of cells in some areas was well advanced and cells outside the vascular cylinder were heavily stained and RNA was present in large amounts in the cytoplasm and in the nuclei.

Cells of the herbicide-treated tissues had a more prominent nucleolus than comparable cells of the water-treated tissues. This apparent increase in volume of the nucleolus is readily seen in the cortical cells of embryos treated with Tordon 22K (Figs. 4.35-4.37) and in those of embryos treated

with Tordon 50D (Figs. 4.38-4.40). Photographs of cortical cells above the root apex in all treatments were taken and enlarged and the diameters of nucleoli were measured. Data were converted to a estimate of nucleoli volume.

Herbicide treatment significantly increased nucleoli volume relative to the controls and Tordon 50D at the concentration used was the more effective of the two herbicides in this regard (Table 4.1).

Table 4.1

Relative mean nucleoli volumes in root cortical cells.  
 $(V = \frac{4}{3} \pi r^3)$ . Population size in brackets)

<u>Tordon 50D</u>	<u>Tordon 22K</u>	<u>Controls</u>
Seed		
1. $381.3 \pm 23.7$ (42)	$412.6 \pm 27.0$ (68)	$273.5 \pm 21.9$ (66)
2. $486.9 \pm 33.0$ (46)	$298.5 \pm 18.6$ (72)	$136.1 \pm 8.3$ (18)
3. $390.5 \pm 26.8$ (56)	$393.6 \pm 30.8$ (39)	$234.5 \pm 15.8$ (60)
4. $474.0 \pm 31.6$ (58)	$351.3 \pm 18.5$ (66)	$219.4 \pm 15.7$ (46)
		$293.7 \pm 31.5$ (26)
		$178.0 \pm 9.3$ (30)
$\bar{x} = 432.7 \pm 15.1$ (202)	$\bar{x} = 359.8 \pm 11.9$ (245)	$\bar{x} = 236.0 \pm 8.9$ (246)

In many cells of the tissues treated with Tordon 50D, nucleoli were not only swollen but appeared to be surrounded by an air space or vacuole within the nucleus (Fig. 4.40 (c)). Malhotra and Hanson (1966) observed an increase in the formation of a DNA-fraction associated with the nucleolus in soybean and cucumber seedlings treated with picloram. These authors suggest this increase in DNA was an indication of active cell division and was followed by excessive ribosomal-RNA synthesis giving rise to growth irregularities and ultimate death of the tissues. Fites et al. (1969) found that a high concentration of 2,4-D

applied to soybean hypocotyls also stimulated DNA synthesis similar to that induced by picloram. This was followed by a considerable increase in ribosomal-RNA. The subsequent proliferation of cells was due to this altered nucleic acid synthesis. They concluded that 2,4-D increased DNA and ribosome synthesis along with messenger-RNA and thus adversely affected those enzymes associated with normal cell division and development. Similar results by other workers relate increases in DNA and cytoplasmic-RNA synthesis with subsequent cell proliferation (West et al. 1960, Key and Hanson 1961, Chrispeels and Hanson 1962, Key et al. 1966, Moreland 1967) and it is now generally accepted that enhanced RNA synthesis is an important effect of the auxin herbicides on plant tissues. Furthermore, Liang et al. (1969) suggested that 2,4-D alters the DNA content of the nucleus so as to cause abnormalities in chromosome behaviour. Chrispeels and Hanson (1962) also found that swelling of the nucleus and a general increase in DNA synthesis preceded ribosomal-RNA production in 2,4-D-treated tissues.

Although under the conditions of this study, no marked changes in cell RNA levels due to herbicide treatment were observed, this does not mean that the herbicides were without effect. The processes of cell elongation and division, which are dependant on nucleic acid synthesis, were affected by the Tordon herbicides and it seemed reasonable to assume that the nucleic acids might also be affected. However, the experimental method used in this study is limited to a qualitative approach to the problem and changes in RNA, particularly at the cell level, may have gone undetected. Nevertheless, the effect of herbicide treatment on the cell nucleoli is of importance and may possibly be associated with Malhotra and Hanson's (1966) increased DNA-fraction found in picloram-treated tissues. There

also appears to be an increase in cytoplasmic-RNA content in day 11 root tissues treated with the herbicides compared with that of the control tissues (cf. Figs. 4.28, 4.29, and 4.30). However, it is difficult to judge whether this increase is on an individual cell basis or results from the greater number of smaller, immature cells in the herbicide-treated root tissues.

#### 4.3 Summary

The effect of Tordon 22K and Tordon 50D on the chemical constituents of germinating seeds of P. radiata was examined using microscopic histochemical procedures. Of the four major chemical investigations, that on ribonucleic acid was the most significant.

Storage or reserve protein structure and content were unchanged throughout the germination period by herbicide treatment. If any alterations did occur, they were undetected by histochemical staining and were likely a result of other physiological responses to herbicide application.

There was a significant effect on carbohydrate content of herbicide-treated material. Root tissues contained large quantities of stored starch and, in some instances, newly synthesized starch. Most of the starch granules were confined to cortical cells outside the zone of proliferating tissue. The elongation and growth of these cells were greatly reduced and hence the demand for high energy sugars of starch metabolism was low. This would account for decreased starch breakdown in the treated root sections. Thus, suppressed cell growth apparently decreases starch catabolism while at the same time newly synthesized starch is being accumulated.

Similarly a decrease in lipid metabolism by herbicide-treated tissues can be related to overall growth suppression. In control tissues, lipid substances decreased over the germination period, their high-energy breakdown products being

rapidly utilized in the normal growth and development processes. However, in the herbicide-treated root tissues, reduced growth probably resulted in less use of lipid reserves and hence a higher content relative to the controls at the conclusion of the experiment. This was thought to be due to a herbicide effect on catabolism rather than on synthesis.

Thus, effects of herbicide treatment on the carbohydrate and lipid contents appear to be the result of the suppressed cell elongation, aberrant cell division and the general decreased vigor of these tissues. The techniques used in this study possibly precluded the association of these abnormalities with changes in nucleic acid contents. However, there was a considerable increase in the volume of the nucleolus and an apparent increase in the amount of cytoplasmic-RNA. These observations relate well with those in the literature and suggest that they could have been primary factors in inducing the growth abnormalities observed.

## CHAPTER 5

EFFECTS OF HERBICIDES ON LEAF AND NEEDLE TISSUE

The results obtained to date in this present study conform well with those reported in the literature. The major effects of both the Tordon herbicides used are on cell division, cell expansion, and on nucleic acid metabolism. However, with minor exceptions, the effects of Tordon 50D and Tordon 22K on P. radiata seeds and early seedling development are identical, and no reasons for the differential effects of these herbicides when applied to older P. radiata seedlings have emerged.

When whole plants of P. radiata and Eucalyptus species are treated with the herbicides, one of the earliest observable responses is an effect on the needles or leaves. With P. radiata, Tordon 50D produces a rapid (within 24 hours) browning of the needles whereas Tordon 22K has relatively little effect (Bachelard and Boughton 1967). With eucalypts, both herbicides produce a rapid wilting of the stem tip and leaves. Hence, the effect of the two herbicides on leaf and stem tissues was examined.

For several reasons, including ease of obtaining replicate material, of reducing the complexity of the material under examination, and of being able to closely control the experimental conditions, the possibility of treating tissue segments in vitro was considered.

### 5.1 Material and Methods

The basal 6 mm of one-year-old P. radiata needles and 6 mm discs from developed first-year leaves of Eucalyptus viminalis Labill. were floated on unbuffered solutions in petri

plates at 30°C under continuous illumination of 130 ft-candles provided by white fluorescent lights. The solutions used were: deionised water as a control; Tordon 22K (25 ppm picloram) and Tordon 50D (25 ppm of picloram and 100 ppm of 2,4-D). Both herbicide solutions were made up in distilled water from commercial formulations.

Tissue samples were removed at daily intervals up to five days, fixed in 3% glutaraldehyde in 0.025M phosphate buffer, dehydrated, and embedded in glycol methacrylate according to Feder and O'Brien (1968). Fixation and dehydration were carried out at 4°C. Sections, 2 $\mu$  thick, were cut with glass knives on a Porter-Blum MT-1 ultra microtome, and mounted on glass slides. P. radiata needle segments were stained with an aqueous solution of 0.05% toluidine blue in a 0.1M phosphate buffer (Feder and O'Brien 1968). Mercury bromophenol blue, a stain highly specific for proteins, was also used to treat the leaf disc material (Pearse 1960).

Sections were examined under a light microscope and photographs taken on a Nikon F 35mm camera.

## 5.2 Results and Discussion

Tordon 50D caused severe shrinkage of the protoplasts of all cells of P. radiata needle segments (Fig. 5.1), these effects being clearly visible after one day. Tordon 22K had no apparent effect even after five days.

Sections of E. viminalis leaf discs after two days' treatment with the herbicide and stained with mercury bromophenol blue, showed that the chloroplasts were affected by both herbicides (Fig. 5.2 (b), (c)). Following treatment with Tordon 22K, the ordered arrangement of chloroplasts is destroyed, and these organelles become condensed and aggregated within the palisade cells. With Tordon 50D, the chloroplast structure breaks down completely leading to an



amorphous aggregation of the protein contents. These results can be interpreted in terms of membrane breakdown. With Tordon 22K, the destruction of the ordered arrangement of the chloroplasts in the palisade cells could be due to the disruption of internal cytoplasmic membranes. With Tordon 50D, the herbicidal effects are even more marked and, in addition to a possible disruption of the cytoplasmic membranes, it appears as if the chloroplast membranes are also destroyed.

These possible effects of herbicides on cell membranes were examined further using an alternating current bridge, similar to the one described by de Plater and Greenham (1959), in which currents of low frequency and of high frequency are passed through the tissue. The resistance of tissue to a low-frequency current (RLF) depends on the integrity of the plasmalemma, and is large in tissues where the cytoplasm and cell membrane are intact. The resistance decreases as the plasmalemma loses its semipermeability and ionic conductivity increases across the membrane (Greenham 1957). Resisting films have less effect on electrical impedance the higher the frequency of the current and hence the resistance of tissue to high-frequency current (RHF) is low regardless of the condition of the tissue (Luyet 1932). The resistance ratio (RLF/RHF), which is more consistent between tissues than either RLF or RHF alone, is high in healthy tissue and approaches unity at death.

Tordon 50D had an extraordinarily rapid effect on P. radiata needle segments which, in this instance, were floated on solutions buffered with M/800 dl-maleic acid buffer adjusted to pH 5 with potassium hydroxide.<sup>1</sup> Resistance ratios fell after only two hours of treatment and most segments were dead by four hours (Fig. 5.3). Segments treated with

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1. Dr. C.G. Greenham, personal communication

Tordon 22K were unaffected. At 48 hours after treatment, the resistance ratio rose in all tissues due to a slightly greater reduction in RHF than in RLF due, probably, to uptake of solutes from the medium.

The resistance ratios fell more slowly for E. viminalis leaf discs (Fig. 5.4). Tordon 50D treatment gave significantly lower ratios after 24 hours, and Tordon 22K treated discs differed from the controls after 72 hours. Changes in the resistance ratio are due to changes in the plasmalemma, and the delayed response in E. viminalis leaf discs compared with P. radiata needle segments is consistent with the microscopic evidence (Fig. 5.2) where the internal cytoplasmic membranes and the chloroplast membranes are those most affected initially.

When stem segments from the tips of E. viminalis branchlets were treated, Tordon 50D again produced a rapid decrease in the resistance ratio, all tissues being dead after 24 hours (Fig. 5.5). Tordon 22K also reduced the resistance ratios but at a slower rate.

To test whether the different response of P. radiata needle segments to Tordon 50D and Tordon 22K was due to the 2,4-D content of the former herbicide, and not to some unknown difference in the commercial formulations, the effects of pure picloram (4-amino-3,5,6-trichloropicolinic acid) and 2,4-D were examined. Stock concentrations (200 ppm) of the pure acids were made up in 0.05M triethanolamine and adjusted to pH 5.0. Dilutions of the stock solutions were made using distilled water. Controls were maintained in a solution of 0.025M triethanolamine.

Picloram alone, at a concentration of 25 ppm duplicated precisely the effects of Tordon 22K (Fig. 5.6) and 2,4-D alone, at a concentration of 100 ppm, duplicated the effects of Tordon 50D. 2,4-D (25 ppm) also killed the tissues rapidly

whereas picloram (100 ppm) gave a much more delayed response. Combinations of the two pure herbicides (Fig. 5.7) confirmed that by far the major response of P. radiata needle segments to Tordon 50D was due to its 2,4-D content.

From these results, the herbicides tested can affect plant cell membranes very rapidly, and the observations are consistent with those from intact seedlings, with P. radiata seedlings being much more sensitive to Tordon 50D than to Tordon 22K whereas eucalypt species appear susceptible to both herbicides.

Effects of herbicides on membrane structure have been reported previously. Atrazine (2-chloro-6-ethylamino-4-isopropylamino-1,3,5-triazine) disrupted both chloroplast and plasma membranes in Phaseolus vulgaris leaves (Ashton et al. 1963 a,b) and in barnyard grass (Hill et al. 1968). Sirmate (3,4-dichlorobenzyl methylcarbonate) affected chloroplast structure in Triticum vulgare L. seedlings (Bartels and Pegelow 1968) while bromacil (5-bromo-6-methyl-3-(1-methyl-n-propyl) uracil) inhibited the full development of chloroplasts in young oat leaves (Ashton et al. 1969).

In addition to these effects of herbicides on chloroplasts and plant cell membranes, both indole acetic acid and naphthalene acetic acid have been shown to affect the properties of plant cell membranes (Sacher 1957, 1959). Segments of bean tissue remained rigid and plump up to 20 days when stored in solutions of IAA or NAA but rapidly became flaccid within 3-4 days when stored in distilled water. On evidence from tissue sections, and plasmolysis experiments, Sacher concluded that the semipermeability of membranes was maintained by these auxins.

2,4-D at a concentration of  $10^{-7}M$  increased the uptake of solutes by Chlorella cells by affecting membrane permeability (Wedding et al. 1959). The uptake of uncharged

particles only, increased, suggesting that the auxin acted primarily on those properties of the membrane which permit passage of uncharged molecules. Higher concentrations of 2,4-D ( $10^{-2}$ M) decreased membrane permeability possibly by altering its composition.

There are indications, therefore, that auxins and auxin herbicides can affect the properties of plant cell membranes. In view of the extremely rapid effect of 2,4-D on the membranes of P. radiata needle segments, and the differential response of this species to picloram and 2,4-D, the possibility exists that effects on plant cell membranes are a primary cause of action of these herbicides.

## CHAPTER 6

### ULTRA-STRUCTURAL CHANGES IN LEAF AND NEEDLE SEGMENTS

Effects of Tordon herbicides on plant cell membranes have not been reported previously, and the evidence presented in the previous chapter indicates that the differential herbicide activity of Tordon 50D and Tordon 22K on P. radiata tissues could be due to differential effects of 2,4-D and picloram on cell membranes within this tissue.

The experiments described here were designed to examine more closely, with the aid of the electron microscope, the effects of Tordon herbicides on plant cell membranes.

#### 6.1 Material and Methods

To ensure uniformity of treatment, in vitro material was again used. Conditions of herbicide treatment were the same as those described previously, namely basal 6 mm segments of one-year-old needles of P. radiata seedlings, and 6 mm discs from fully developed leaves of one-year-old E. viminalis seedlings were floated on unbuffered solutions in petri plates. Deionised water was used for control tissues while herbicide solutions were applied as Tordon 22K (25 ppm picloram) and Tordon 50D (25 ppm of picloram and 100 ppm of 2,4-D). The temperature was maintained at 30°C and the tissues received constant illumination of 130 ft-candles from a bank of white fluorescent lights.

Needle segments of P. radiata were removed at 2,4 and 8 hour intervals after the start of treatment, and E. viminalis leaf discs after three and six days.

Prior to fixing material in glutaraldehyde, sections were blotted dry and resistance ratios were checked on the alternating-current bridge. It is sufficient to note that these ratios corresponded to those found previously for tissues in similar treatment and time periods.

Small portions (approximately 1 mm) of leaf and needle material were fixed in 1.5% glutaraldehyde in 0.025M phosphate buffer, pH 6.8, and vacuum infiltrated in Thunberg vessels for two hours at room temperature.<sup>1</sup> Tissues were transferred to a 6% glutaraldehyde solution in phosphate buffer for 12 hours. After several washings in buffer, the material was post-fixed with 1% osmium tetroxide in 0.025M phosphate buffer for two hours. Osmium tetroxide is a slow penetrating fixative but ideal for preserving intact the ultrastructural detail of plant cells (Kay 1961).

Dehydration and embedding in araldite was carried out at room temperature according to the following schedule.

- (1) After post-fixing in osmium tetroxide, sections were rinsed well in distilled water, and placed in 1% uranyl acetate in 15% ethyl alcohol for half an hour.
- (2) Tissue sections were dehydrated in a series of 1% uranyl acetate in 25, 50 and 75% ethyl alcohol, half an hour in each solution. They were then left overnight in 100% ethyl alcohol.
- (3) Sections were placed in a mixture of ethyl alcohol: propylene oxide (50:50 v/v) for three hours and transferred to 100% propylene oxide for an additional three hours.
- (4) An araldite mixture consisting of 20 mls each of epoxy hardener (HY964) and araldite M epoxy resin (CY212) and containing 1.5 mls of dibutyl phthalate and 0.6 mls accelerator (DY064) was prepared. Tissues were placed

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1. Dr. M. McCully, personal communication

in a 25% araldite mixture in propylene oxide solution for three hours, in 50% araldite in propylene oxide overnight, and finally in 75% araldite in propylene oxide for four hours.

- (5) The last step was to place the tissue sections in 100% araldite in small plastic trays, the araldite being hardened for five days at 60°C.

After the blocks had hardened they were trimmed initially with glass knives, and sections cut with a diamond knife on a "Reichert Om U2" ultramicrotome. Sections which refracted silver/gold to grey interference colours after flattening (approximately 600-1000 Å thick) were mounted on carbon-coated 200-mesh copper grids.

By staining sections with heavy atoms such as lead, membranous structures become readily visible with the electron microscope. Grids with P. radiata and E. viminalis sections were treated with saturated uranyl acetate for 30 minutes and stained five minutes with lead citrate according to the method of Reynolds (1963).

Tissue sections were examined and electron micrographs taken on an electron microscope, JEM model T6.

## Results 6:2 Pinus radiata needle segments

### 6:2.1 Effects on nuclear and plasma membranes

The differential permeability of cells to solutes is due to two surface membranes, the tonoplast or inner plasma membrane between the protoplasm and vacuoles, and the plasmalemma, external to all cell contents and normally adpressed against the cell wall (Weier and Thomson 1962).

Following treatment with Tordon 50D there was no evidence of membrane damage in P. radiata cells after two hours, but by four hours effects on membranes were quite clear, and after eight hours many of the cell membranes were

completely disrupted.

After four hours of Tordon 50D treatment, the cytoplasm of phloem parenchyma cells in the vascular region was relatively unaffected although the plasmalemma in most cells had pulled away from the cell walls (Fig. 6.1 (a)). Nuclei of these cells were intact but irregular in shape, and their contents greatly condensed (Fig. 6.1 (b)).

This observed effect on the plasmalemma may be due to disruption of the integrity of the plasmalemma and could be responsible for the earlier observations of decreased resistance ratios of Tordon 50D-treated needle segments after four hours. Cells taken from a similar region of control and Tordon 22K-treated needle tissues after four hours appeared completely healthy and unaffected by treatment (Fig. 6.1 (c)).

After eight hours of treatment with Tordon 50D, the effects on the cell membranes were even more noticeable. The plasmalemma of cells in the vascular tissue was markedly altered and in many cells, had pulled away completely from the cell wall, and the remnants were lying towards the centre of the cell. These affected cells were void of cytoplasm (Fig. 6.2 (a)). Cell structures, presumably mitochondria and plastids, lying between the plasmalemma and the tonoplast were highly vacuolated, and ultimately became unrecognizable membranous structures (Fig. 6.2 (b)).

Destruction of nuclei was evident in all phloem parenchyma cells of the vascular tissue. The nuclear contents coalesced into electron-dense, plate-like bodies (Fig. 6.2 (c)) which gradually broke down, leaving the nucleus void of any material but still bounded by the nuclear membrane (Fig. 6.2 (b)).

With the destruction of the nucleus and cytoplasm and the breakdown of the plasma membranes, death of the cells in



the vascular region was complete.

Tordon 22K caused no damage to nuclei or membrane integrity after eight hours of treatment (Fig. 6.2 (d)). This is consistent with the results from the alternating current bridge where resistance ratios of Tordon 22K-treated needle segments maintained values equal to the controls.

### 6:2.2 Effects on chloroplast structures

The functional chloroplast unit is a compartmented disc or thylakoid body formed of two parallel, flattened membranes, joined at their margins and enclosing an electron transparent space, the loculus (Weier and Thomson 1962, Thomson and Weier 1962, Menke 1962). Thylakoids are found in lamellate structures or grana which are composed of individual thylakoids lying parallel to one another. These granal lamellae contain chlorophyll and are the site of the light phase of the photosynthetic process (Wolken 1959, Menke 1962). Adjacent grana are connected by a system of flattened tubular thylakoids or frets extending irregularly from the thylakoid units. Both the granal and intergranal fretwork systems are embedded in a matrix, the stroma, containing soluble enzymes of the photosynthetic process (Weier et al. 1963). Chloroplasts may also contain lipid globules and starch grains seen as large electron-clear areas.

Chloroplasts of P. radiata have extremely long lamellated grana and the area of the intergranal fretwork system is relatively small (Fig. 6.3 (a)).

Although Tordon 22K has no effect on nuclei or plasma membranes, it is not without an effect on the chloroplasts of P. radiata needle segments. The herbicide caused an overall swelling of the chloroplasts in palisade cells after eight hours of treatment, changing their shape from discoid

to oval, but did not alter their normal peripheral distribution about the cell (Fig. 6.3 (b)). The internal thylakoid membranes were disrupted after eight hours of herbicide treatment when they became greatly swollen, creating large, electron-transparent areas between adjacent thylakoids (Fig. 6.3 (c)). There was no systematic unstacking of grana compartments but a random and irregular swelling of individual thylakoids. In some grana, thylakoids retained their flattened shape and remained parallel to one another, while in other areas of the same chloroplast, thylakoids separated, increasing their volume without any rupture of their bounding membranes. Some thylakoid membranes increased in thickness, becoming diffuse and gradually disintegrating within the chloroplast (Fig. 6.3 (c)). The chloroplast membrane itself was apparently unaffected by herbicide treatment but the stroma or photosynthetic matrix appeared to be largely dispersed compared with control chloroplasts.

In Tordon 50D-treated needle tissue there was a gradient of chloroplast responses after eight hours of treatment. Chloroplasts of the palisade cells were swollen and had greatly disorganized granal and fretwork systems. Large clear areas were created between grana, and individual thylakoids of grana were also swollen. Stromal material was, in most instances, completely absent (Fig. 6.4 (a)). In the extreme herbicide response, organelles, which appear to be chloroplasts, although little lamellate structures remain, were found between the plasmalemma and tonoplast (Fig. 6.4 (b)). These structures were found in phloem cells of the vascular region, the first cells affected by Tordon 50D. They contained starch grains and large membranous bodies which probably originated as swollen thylakoid compartments. These chloroplasts were void of stromal material and had long ceased to function. Their internal membranes were greatly distorted and thickened, due possibly to several thylakoid membranes adhering together.

### 6:3 Eucalyptus viminalis leaf discs

The response of E. viminalis leaf tissue to herbicide treatment was observed over a period of days rather than hours as with P. radiata needle segments. Measurements with the alternating-current bridge showed that Tordon 50D had an earlier toxic action on leaf material than Tordon 22K. After one day of treatment with Tordon 50D, resistance ratios were significantly less than the controls and after three days, had fallen to a level indicative of irreversible damage to the plasmalemma and death. Tordon 22K-treated leaf discs, however, maintained high resistance ratios up to day 3. After this period, resistance ratios gradually dropped as injury to the cell membranes occurred.

Chloroplast structures of E. viminalis have granal-fretwork systems similar in structure and function to those described for P. radiata. Large starch grains may be present in the chloroplasts and in addition, they regularly contain oval, electron-dense bodies presumably of lipid nature. These globules vary in size and are randomly distributed in the stroma (Fig. 6.5 (a)).

When sections of herbicide-treated leaf discs were examined under the electron microscope, it could be seen that chloroplast and plasma membrane integrity as well as chloroplast distribution in the cell were affected by treatment. After 3 days treatment with Tordon 50D, distortion of the cell membranes was well advanced and there was a gradient of moderate to severe chloroplast disruption in most cells. The plasmalemma had pulled away from the walls of many palisade and mesophyll cells, and cytoplasm of these cells was almost completely absent. Chloroplasts in these cells were enlarged and swollen, and their internal membranous network was in various stages of disarray (Fig. 6.5 (b)). Individual thylakoid membranes had separated, causing the unstacking and disruption of grana. Thylakoids were distorted

from their normal flattened disc shape to large oval vesicles, beginning usually with marginal thylakoids and continuing throughout the granum (Fig. 6.5 (b)). This alteration of the grana and fret membranes occurred before the eventual rupture of the chloroplast envelope and, in extreme cases of herbicide damage, chloroplast contents had spilled into the cell to mix with the contents of other chloroplasts and with what little cytoplasm remained. Owing to breakdown of the tonoplast in some cells, chloroplasts were not always found along the wall but aggregated in the cell where their limiting membranes became ruptured (Fig. 6.5 (c)).

In some cells, particularly in the palisade layer, the internal chloroplast membranes were relatively unaffected although the chloroplasts were greatly swollen and contained enormous starch grains. There was some evidence of damage to the plasmalemma and tonoplasts in these cells (Fig. 6.6 (a)). Starch was absent from chloroplasts from similar cells after six days of treatment. Tonoplast membranes were lacking in most cases, and the chloroplasts were swollen and clumped together in the cells (Fig. 6.6 (b)). After six days of treatment, some mesophyll and palisade cells contained structureless masses with remnants of granal and fret thylakoids. Outer limiting chloroplast membranes had completely broken down.

Tordon 22K-treated leaf discs were largely unaffected after three days of treatment. Cell membranes were intact and the peripheral distribution was not greatly altered. This contrasts with results previously found with material examined under the light microscope where herbicide effects were visible after two days of treatment. There was evidence, however, in three-day tissues, of an internal chloroplast response to herbicide treatment consisting mainly of a slight

swelling of individual thylakoids associated with grana and a general distortion of fretwork membranes. By the end of six days of treatment, herbicide damage was extensive. Cytoplasm was either totally absent or greatly vacuolated in the mesophyll and palisade cells, and the plasmalemma had pulled away from the walls (Fig. 6.7 (a)). Cell membranes at this stage had withdrawn from the walls towards the cell centre, moving cell organelles with them.

Chloroplasts were also severely affected after six days of Tordon 22K treatment. Mesophyll and some palisade cell chloroplasts were abnormally swollen, contained large starch granules and lacked stroma material. Some granal thylakoids retained their parallel alignment while others were swollen and the grana disrupted. In some instances, the double-membrane of the chloroplast envelope had broken, permitting chloroplast contents to escape into the cell (Fig. 6.7 (b)). Other palisade cells contained chloroplasts along the walls but with evidence of internal membrane damage (Fig. 6.7 (c)). Starch grains were present in some chloroplasts of Tordon 22K-treated leaf discs after six days but these were not as common as in the control tissues. Most chloroplasts of Tordon 22K- and Tordon 50D-treated material which still retained some semblance of structure also contained lipid globules but there was no apparent correlation between lipid content and herbicide treatment.

Plasmalemma and tonoplast membranes were intact, and all chloroplasts were unaltered in control leaf discs after six days (Fig. 6.7 (d)).

#### 6:4 Discussion

Examination of P. radiata needle segments and E. viminalis leaf discs using the electron microscope confirms

and extends the observations obtained previously using the light microscope and the alternating-current bridge.

Tordon 50D rapidly destroys the integrity of the nucleus, and the plasmalemma and tonoplast of cells in P. radiata needle segments. The effect on the membranes is particularly rapid, being quite noticeable within four hours of treatment and advanced after eight hours. The granal and fretwork thylakoid membranes of the chloroplasts are also disrupted by Tordon 50D.

Tordon 22K has no visible adverse effects on the plasmalemma or the tonoplast within the treatment period examined here and, as far as can be observed, the cells remain alive and healthy. However, chloroplasts are affected by Tordon 22K, the first visible symptom being the breakdown of the lamellate structure and, ultimately, the complete collapse of the chloroplasts.

In contrast to the P. radiata tissue, E. viminalis leaf discs are affected similarly by Tordon 50D and Tordon 22K although the response of this tissue to herbicide treatment is considerably slower than for P. radiata tissue. Also the response to Tordon 22K is much slower than to Tordon 50D.

After three days of treatment with Tordon 50D, the chloroplasts of the eucalypt leaf discs are swollen and, in some cases, their internal membrane structure is completely destroyed. The tonoplast is ruptured in some cells resulting in the aggregation of chloroplasts in the centre of the cell and the liberation of chloroplast contents in this region at the final disintegration of the chloroplast structure. As with P. radiata tissues, the plasmalemma is dissociated from the cell wall of many cells of the palisade and mesophyll layers. Treatment with Tordon 22K produces very similar effects but only after a treatment period between three and

six days. In previous work using the light microscope (Chapter 4) a response to Tordon 22K was observed after two days.

Effects on chloroplasts similar to those described here may be caused by environmental factors. Weier et al. (1963) observed that the movement of plants from darkness to light, and also zinc deficiency, can cause swelling of internal chloroplast membranes similar to that induced by atrazine. Hill et al. (1968) reported an effect of mineral deficiency in modifying the grana and fretwork structure of chloroplasts, but atrazine gave a more drastic and long lasting response. In this present study, the chloroplasts of the control tissues showed no swelling or distortion of the internal thylakoid membranes, and the plant cell membranes appeared unaffected. The results described for Tordon 50D- and Tordon 22K-treated tissues therefore can be attributed with confidence to herbicide treatment.

Detailed reports in the literature of other herbicides affecting the structure of chloroplasts and plasma membranes describe similar responses to those observed here for the Tordon herbicide treatments. Bromacil inhibited the full development of grana and fretwork membranes in young chloroplasts of oat leaves (Ashton et al. 1969). Eight days after treatment, fret vesicles and granal thylakoids were swollen and the double chloroplast membranes had separated and ruptured. Ashton et al. (1963 a,b) found that atrazine applied to Phaseolus vulgaris leaves caused a general collapse of cells, and this was thought to be due to changes in cell membranes causing the leakage of cellular contents. Chloroplast structures in some cells were also affected, and the tonoplast ruptured. Palisade cells in particular, were greatly vacuolated with swollen, rounded chloroplasts. With the breakdown of the tonoplast, these chloroplasts became

aggregated into groups within the cells. Within 30 hours of treatment, individual chloroplasts of leaves treated with atrazine in the light showed some herbicide response. Swelling of granal compartments beginning with the end thylakoids resulted in a completely disorganized lamellate system. The final stage was the total collapse of the chloroplast envelope and the release of the stroma matrix into the cell vacuole. Ashton et al. (1963 b) suggested that these responses were not due to the herbicide itself, but to a toxic substance produced by the interaction of atrazine and light, since the effects were absent from plants treated with atrazine in the dark. It was the toxic substance that was thought responsible for altering the chloroplast membrane properties, and accounted for the change in chloroplast shape and their eventual disintegration.

Hill et al. (1968), examining atrazine-treated leaves of barnyard grass, found chloroplast lamellae swollen and disorganized after two hours of treatment. The chloroplast envelope and thylakoid membranes ruptured after four hours treatment with 5 ppm of atrazine. Swelling of the granal and fretwork membranes occurred prior to their rupture, indicating these membranes have a certain amount of plasticity and stretch potential.

The herbicide pyriclor (2,3,5-trichloro-4-pyridinol) also caused the disruption of chloroplast structure when applied to tobacco leaves (Geronimo and Herr 1970). Chloroplasts examined 144 hours after the tissues were treated with 40 ppm herbicide showed severe swelling of end compartments of grana. Inner thylakoids were also swollen, and the intergranal fretwork system almost completely vesicular. Final effect of herbicide treatment was the complete disintegration of the chloroplast envelope and scattering of the contents.



Anderson and Schaelling (1970) suggested that the swelling of chloroplasts of bean leaves treated with pyrazon (5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone) was due to an alteration in membrane permeability, permitting the chloroplasts to swell without rupturing. Granal membranes became swollen and ultimately disintegrated as a result of herbicide treatment. There was also an increase in the number of lipid globules in chloroplasts of treated leaves.

Plastids in bean leaves treated with the herbicide CMU (3-(4-chlorophenyl)-1,1-dimethylurea) had developed fewer but larger grana 24 hours after treatment compared with controls (Klein and Neuman 1966). Thylakoid membranes of the fretwork system were almost completely absent.

The earliest effect of paraquat (1,1'-dimethyl-4,4'-bipyridylium-2A) on honey mesquite leaves was the breakdown of the plasmalemma in mesophyll cells followed by the rupture and ultimate disintegration of the chloroplast membranes (Baur et al. 1969).

It may be observed, therefore, that the effects of both picloram and 2,4-D on chloroplast structure as reported here are similar to those exhibited by a wide variety of herbicides. With many of these other herbicides the major toxic action is thought to be on some part of the photosynthetic process, hence the emphasis on studies of chloroplasts. It is not suggested here that the herbicidal properties of picloram and 2,4-D are due primarily to their effects on chloroplast structure but rather that the effects on chloroplast structure may be a manifestation of a wider effect of these herbicides on plant cell membranes.

One of the few reports on the effects of 2,4-D on plant cell membranes has recently been made by Hallam (1970) who observed herbicide-induced changes in the morphology and internal membrane structure of primary leaves of Phaseolus

vulgaris. Four hours after a  $1.6 \times 10^{-3}M$  2,4-D treatment, the plasmalemma and endoplasmic reticulum as well as the outer chloroplast membranes were disrupted in cells of the epidermis, palisade, and mesophyll layers. After eight hours, the plasmalemma had receded from the cell walls and chloroplasts were further distorted, with a marked increase in vesicles associated with the stroma and invaginations of the inner chloroplast membrane into the stroma. The effects of 2,4-D on membrane structure were further pronounced 24 hours after treatment with many cells appearing plasmolysed and with ruptured chloroplasts containing numerous swollen thylakoid membranes.

The gradual disappearance of starch from herbicide-treated chloroplasts as duration of treatment increased was observed by Ashton et al. (1963 b) and Hill et al. (1968), and would suggest that the lack of starch in E. viminalis leaf discs after six days, particularly those treated with Tordon 50D, is directly the result of herbicide treatment. Starch has also been found to disappear from chloroplasts in leaves treated with pyrazon (Anderson and Schaeffer 1970), with pyriclor (Geronimo and Herr 1970), and with 2,4-D (Hallam 1970).

The amount of stromal material in herbicide-treated chloroplasts of both needle segments and leaf discs decreased as treatment time increased and herbicide response became more acute. Lamellae were difficult to observe in control chloroplasts because of the very dense stroma but were clearly visible in herbicide-treated chloroplasts. Sirmate (Bartels and Pegelow 1968) and amitrole (3-amino-1,2,4-triazole) (Bartels et al. 1967) have been reported to alter the stroma composition of chloroplasts by reducing the ribosome and fraction-1-protein content. Cytoplasmic ribosome content was unaffected in both cases. Bartels and Pegelow (1968) suggested that, for sirmate, the general lack of internal chloroplast membranes may be caused by the loss of chloroplast ribosomes.

## CHAPTER 7

EFFECTS OF HERBICIDES ON INTACT SEEDLINGS

In the previous two chapters, it was suggested that effects on plant cell membranes could be a mechanism of herbicide action of 2,4-D and picloram. The evidence for this was obtained from isolated tissue segments and, although such material can prove extremely useful in evaluating plant response to particular treatments, the responses may not be identical with those occurring in intact plants. The complex inter-relationships of growth and development that exist between different parts of a plant may modify the response of the plant to the treatment. It was desirable, therefore, to examine the effects of 2,4-D and picloram on intact P. radiata and eucalypt seedlings with particular attention being paid to the effects on cell membranes.

7:1 Materials and Methods

One-year-old P. radiata and 6-months-old E. camaldulensis Dehnh. seedlings were used in this study. E. camaldulensis seedlings were treated here instead of E. viminalis because of the availability of suitably-sized seedlings, and the similarity between eucalypt seedlings in their response to herbicides.

Both pine and eucalypt seedlings were kept in a glasshouse and exposed to daylengths of 16 hours. Normal daylengths were supplemented with fluorescent and incandescent lights. All seedlings were growing actively at the time of treatment.

Stock solutions of analytical grade picolinic acid and 2,4-D were made up in 0.1M triethanolamine solutions. The treatment solutions were 0.2% picloram and 0.8% 2,4-D,

each solution containing 0.05M triethanolamine and 0.7% Decol T/70 (triethanolamine dodecyl benzene sulphonate) as surfactant. Control solutions were 0.05M triethanolamine containing 0.7% Decol T/70. The picloram concentration used is commonly applied in field practice (low volume applications); the 2,4-D concentration is somewhat lower than that used in the field.

Herbicide or control solution was applied as a 50 $\mu$ l droplet directly to the apical growing tip of the pine seedlings, care being taken to avoid run-off of the solution from the point of application. Growing tips, and stem sections taken approximately 5 cms below the tip, were removed 1,3 and 7 days after treatment. Each treatment was applied to three seedlings for each time period.

With E. camaldulensis seedlings, 20 $\mu$ l and 80 $\mu$ l droplets of the herbicide solutions were applied to one leaf of the fourth leaf pair from the shoot tip. For control seedlings, only 80 $\mu$ l droplet applications were made. Droplets were spread over the surface of the leaf with the aid of a fine brush. Sections of the extreme stem tip, free of leaf tissue, were removed after 1,3,7 and 15 days of treatment. All treatments were applied to two seedlings for each time period.

All segments, being removed from the seedlings, were cut under a 1.5% glutaraldehyde solution in 0.2M phosphate buffer prior to fixation and embedding.

P. radiata tips and stem segments were fixed, dehydrated and embedded in polyester wax as described earlier (Chapter 3). Sections, 10 $\mu$  thick, were cut on a cryostat at 8°C and mounted on glass slides. E. camaldulensis tissues were fixed, dehydrated, and embedded in glycol methacrylate as outlined in Chapter 5, after which 2 $\mu$  sections were cut on a Reichert OM U2 ultramicrotome. All sections were stained with 0.05% toluidine blue.

For examination under the electron microscope, segments were fixed, dehydrated, and embedded in araldite according to the schedule outlined in Chapter 6, and sections (approximately 600-1000<sup>0</sup>Å thick) were cut on the ultramicrotome using a diamond knife and collected on copper grids. Sections were stained with uranyl acetate followed by brief staining with lead citrate, and viewed under a JEM model T6 electron microscope.

## 7:2 Results

### 7:2.1 Gross Morphology

Effects of picloram and 2,4-D on P. radiata seedlings are similar to those caused by Tordon 22K and Tordon 50D respectively. Within 24 hours after treatment with 2,4-D, the tip needles were brown and dying. Picloram-treated and control seedlings were unaffected. After three days 2,4-D treatment, the stem tip as well as the tip needles were browned and a sticky exudate was present at the tip. Stems were bent slightly. One week after treatment, 2,4-D injury was well advanced with most tips being dead and stems bent.

The tips of P. radiata seedlings treated with picloram were slightly swollen after three days and needles at the point of application were yellow-green in colour. These effects were more pronounced after seven days, but in spite of swelling of the tip and curling of some tip needles, the tip was alive and active.

The effects of picloram on E. camaldulensis seedlings were more pronounced than those of 2,4-D. After one day of treatment with 0.2% picloram, both the 20µl and 80µl applications caused the bending of the stem tip and some curling and cupping of the upper leaves (Fig. 7.1 (a), (b)). These effects became more pronounced as the time from treatment increased (Fig. 7.1 (c)). The morphological

response of E. camaldulensis seedlings to 2,4-D, although delayed and less intense, was similar to that induced by picloram (Fig. 7.2). Difference in the time of response to the two herbicides is possibly due to differences between their mobilities in the plant as found by Merkle and Davis (1967) for picloram and 2,4,5-T. The movement of picloram was greater than that of 2,4,5-T regardless of the moisture stress state of the plant.

Control seedlings were unaffected by treatment except for the treated leaf which became mottled over portions of its area. This was presumably due to the toxicity of triethanolamine at the point of application.

For the herbicide concentrations used and under the experimental conditions employed, these results show:

- (i) A rapid toxic effect of 2,4-D on P. radiata seedling tips, and a relatively non-toxic effect of picloram, although this herbicide does lead to swelling of the shoot apex and chlorosis of the treated needles.
- (ii) A rapid effect of these herbicides, particularly picloram, on the stem and leaves of E. camaldulensis seedlings some distance from the point of application of the herbicide.

#### 7:2.2 Anatomical effects on P. radiata tissues

Some effects of herbicides on the anatomy of P. radiata seedlings viewed at the light microscope level are illustrated in Figs. 7.3 and 7.4. Picloram, at one and seven days after application, had no obvious effects on stem tissues taken approximately 5 cms below the shoot apex (Fig. 7.3 (b) and Fig. 7.4 (b)). There is however, some evidence of increased cambial activity in the day 7 material. 2,4-D, on the other hand, markedly stimulated cambial activity after one day (Fig. 7.3 (c)), and by seven days, there was a proliferation of cells in and around the vascular cambial region,

and a general disorganization and breakdown of cells in this region (Fig. 7.4 (c)).

Tissue sections of tips treated with 2,4-D examined under the electron microscope also showed the greatest response to treatment by cells in and around the vascular region. In tissues taken three days after treatment, the protoplasts of long, narrow cells having irregular walls, which were presumed to be cambial initials and their immediate derivatives, were disrupted and lay against one wall or in the centre of the cell (Fig. 7.5 (a)). The cytoplasm in these cells was dense and large electron-clear areas, possibly starch grains, were abundant.

In other cells in the vacular cambium region, membranes were similarly distorted and withdrawn from the walls. The cytoplasm was disrupted and a large number of cell organelles, possibly mitochondria, were in various stages of breakdown (Fig. 7.5 (b)). Nuclei of these cells were disrupted internally although nuclear membranes appeared intact.

Larger cells in the same general area of the tissue, possibly ray parenchyma cells, showed similar membrane disruption, disintegration of the cytoplasm and the occurrence of numerous vesicles associated with the membranes (Fig. 7.5 (c)) as found in other cells of the vascular region.

The picloram-treated tissues differed markedly from those receiving 2,4-D. After three days of treatment, all cells in and around the vascular cambium contained intact nuclei with prominent nucleoli (Fig. 7.6 (a)) and cell membranes were apparently unaffected (Fig. 7.6 (b)).

Seven days after treatment with 2,4-D, the disruption of cell membranes and breakdown of nuclei in the cells of the seedling tips were well advanced. Cells of the vascular region were extensively damaged with nuclei and cytoplasm not present in many cells. The cell membranes were highly distorted and

vesicular, and cytoplasmic organelles were vacuolated (Fig. 7.7 (a), (b), (c)). The internal structure of many nuclei was disrupted but the nuclear membrane remained intact (Fig. 7.7 (d)).

After seven days, tissues of the seedlings treated with picloram were still relatively unaffected. The nuclei in general appeared functionally intact (Fig. 7.8 (a)), the cells retained their dense cytoplasm and the cytoplasmic organelles were not vacuolated (Fig. 7.8 (b)). Some cambial cells and vascular parenchyma were observed to have distorted and ruptured cell membranes, and damaged nuclei (Fig. 7.8 (c)). Such effects, however, were relatively rare and, after seven days, not nearly as marked as in 2,4-D-treated material after three days.

All sections from the control tissues showed healthy, intact cells and cell organelles with abundant cytoplasm (Fig. 7.9 (a)) and intact nuclei (Fig. 7.9 (b)).

### 7:2.3 Anatomical effects on *E. camaldulensis* tissues

Although *E. camaldulensis* seedlings showed a rapid morphological response to herbicide treatment, particularly picloram, in wilting of the stem tips and upper leaves, this response is not so evident in sections of stem tissue viewed with the light microscope (Fig. 7.10). There are indications that the health and integrity of some cells in the tissues, particularly the cortical, and possibly the cambial cells, have been disturbed by herbicide treatment but, at this level of magnification, it is not possible to observe just what might have occurred.

Even under the electron microscope, the effects of herbicides on *E. camaldulensis* tissues were not as obvious as they were in *P. radiata* sections. However, effects, similar in nature to those occurring in 2,4-D-treated *P. radiata* seedlings, were observed. Cells in and around the vascular



cambium were again those most affected, and in tissues taken three days after picloram application, disruption of cell membranes was apparent (Fig. 7.11 (a)). After seven days, these effects by picloram were more pronounced with the membranes in some cells being greatly distorted and ruptured, and cytoplasm absent (Fig. 7.11 (b)).

2,4-D, after seven days, appeared to induce even greater damage to the cambial and phloem cells, although the general response is identical and includes rupturing and distortion of the membranes, absence of cytoplasm, high occurrence of vesicles attached to the membranes or lying within the cell, and vacuolation of cell organelles (Fig. 7.11 (c), (d)).

Although a 20 $\mu$ l application of 2,4-D had no clear morphological effects on E. camaldulensis seedlings until seven days after application, sections taken from seedlings after three days contained some cells in which cell membranes were disrupted and possessed a number of associated vesicles.

These effects of herbicides were not observed in all cells of the vascular region in any particular section but they were stimulated by herbicide treatment, and appear to be a result of it. Typical cells from a control section are shown in Fig. 7.12 (a).

An additional possible effect of herbicide treatment was a marked increase in the prominence of some nucleoli (Fig. 7.12 (b), (c)) and it is of interest to note that Malhotra and Hanson (1966) observed an increase in the DNA-fraction of nucleic acids associated with the nucleolus after picloram treatment.

### 7:3 Discussion

The effects attributed in this chapter to picloram and 2,4-D toxicity on P. radiata and eucalypt seedlings were

observed under a rigid set of experimental conditions. In order to accurately assess these results and apply them to a wider field of herbicide knowledge, a brief discussion of factors affecting herbicide toxicity is necessary.

The toxicity of any herbicide depends on numerous interrelated factors and a statement concerning absolute herbicide toxicity is exceedingly difficult to make. Toxicity is a variable herbicide property and can not simply be regarded in positive or negative terms. Sutton (1958) observed that any plants may be killed by large enough applications of the chemicals 2,4-D and 2,4,5-T, but to achieve a degree of selectivity between wanted and unwanted vegetation requires the consideration of many related variables including form and concentration of herbicide, the manner of application, physiological state of the plants, various environmental factors and the response to be measured and its time of measurement.

Herbicide form is important when considering toxicity. The ester formulations of 2,4-D are the most effective in the control of woody vegetation (Sutton 1958). Further, Johanson and Muzik (1970) have reported that the isopropyl ester of 2,4-D was more toxic to wheat plants than either the triethanolamine salt or the solubilized acid forms applied at the same concentration. This effectiveness may be partly related to uptake. Lenard et al. (1966) found the uptake of 2,4,5-T in plants was greatest with the ester formulation and least with the triethanolamine salt.

Increasing the concentration of herbicide applied generally results in greater toxicity but other factors are also involved. Fisher et al. (1968) treated Phaseolus vulgaris seedlings with picloram and found that with younger plants, lower herbicide concentrations produced adverse effects similar to those observed in older seedlings treated at higher

concentrations. The most effective kill of rabbit bush by 2,4-D occurred when plants were nearly in full leaf, but the same concentration applied when the plants were in bloom had little or no effect (Laycock and Phillips 1968).

Toxicity of a particular herbicide concentration also depends on the manner of application. Seeds left in continuous contact with CDAA (2-chloro-N,N-diallylacetamide) failed to germinate, but when they were pretreated for 24 hours with the same concentration and then planted out in soil, germination occurred after 11 days (Sasaki et al. 1968 a). Kozlowski and Torrie (1965) showed the toxicity of a herbicide was greatest when plants were maintained in direct contact with the herbicide, intermediate when the herbicide was incorporated into the soil, and least when applied only to the soil surface.

Environmental conditions also affect the degree of toxicity achieved by a herbicide application. High air temperatures increased the toxicity of the triazine herbicides although the effects of temperature varied greatly among the herbicides used (Kozlowski et al. 1967). The highest experimental temperature used (30°C) had more effect on simazine toxicity than did a higher herbicide concentration applied at a lower temperature. Sasaki and Kozlowski (1968 b) reported that herbicides such as atrazine, simazine and monuron which specifically inhibit the photosynthetic process, showed increased toxicity with increasing light intensity. Soil properties have also been shown to affect herbicide toxicity e.g. soil temperature (Cords 1966), moisture content (Laycock and Phillips 1968), and soil type and organic content (Herr et al. 1966, Merkle et al. 1967).

The parameter selected as an indication of toxicity and the time scale for response measurement also determine the toxicity rating of a herbicide. Roots are apparently more

susceptible to 2,4-D and picloram applications than the above ground portions of the plant (Rojas-Garciduenas et al. 1962, Johnson 1967, Kozlowski and Sasaki 1968 a). Germination may be unaffected by some herbicides at particular concentrations but the subsequent seedling mortality and development may demonstrate herbicide toxicity (Sasaki et al. 1968).

Bachelard and Johnson (1969) found no effect of Tordon 50D and 2,4,5-T soil-surface applications on P. radiata seed germination. However seedling development and survival were affected. The time of response measurement after treatment also influences herbicide toxicity rating. Kozlowski and Torrie (1965) observed that when ipazine herbicide was incorporated into the soil, more P. resinosa seeds died in the last 20 days of the experiment than in the first 90 days. Bachelard and Johnson (1969) demonstrated high toxicity of picloram to pine seedlings four months after a soil-surface application and that even after seven months various degrees of toxicity persisted in the soil. The toxicity of 2,4,5-T in the soil was greatest up to two months after treatment with some slight toxicity remaining three months after treatment.

These considerations of factors affecting the concept of herbicide toxicity indicate the difficulty in assessing plant response to herbicide treatment. The results presented in this chapter occurred under specific conditions and should not be extrapolated to the general behaviour of the herbicides on P. radiata and eucalypt seedlings. Nevertheless, under the conditions imposed, the effects of picloram and 2,4-D on intact seedlings are consistent with the previous observations of in vitro reactions of tissue segments to these herbicides (and to Tordon 22K and Tordon 50D). Both herbicides stimulated cell division in stem tips of P. radiata seedlings and this increase in cell division was almost certainly responsible for the swollen apices of seedlings treated with picloram.

Picloram, however, does not kill P. radiata seedling apices; 2,4-D does, and it is difficult to explain the very rapid toxic effect of 2,4-D in terms of increased cell division.

Similarly, with E. camaldulensis seedlings, both herbicides, when applied to a single leaf four leaf pairs below the growing tip, induced a rapid wilting of the stem tip and uppermost leaves. These effects were induced by 40µg of picloram within 24 hours. Even after seven days, there was no obvious stimulation of cell division by herbicides in the eucalypt stem tips. It would appear, therefore, that the herbicides are having a much more direct phytotoxic effect than one mediated through unregulated cell division.

2,4-D rapidly browns and kills P. radiata stems. Both 2,4-D and picloram rapidly wilt stem tips and leaves of eucalypt seedlings. Both these responses could be caused by a severe and rapid loss of water by the cells. Evidence from both tissue segments and intact seedlings suggests that the herbicides used in this study can have a direct and severe effect on plant cell membranes leading both to a rapid and irreversible loss of water from affected cells and destruction of the complete metabolic machinery within these cells.

## CHAPTER 8

DISCUSSION

In this chapter an attempt is made to draw together the more important aspects of the observations made in this study, and to relate them to each other and to recent reports in the literature.

Of the plant constituents examined by histochemical techniques (Chapter 4) the ribonucleic acid study seems the most significant and the apparent changes in RNA levels in herbicide-treated tissues could account for many of the growth abnormalities recorded (Chapter 3). Root tissues were more affected by treatment with Tordon 22K and Tordon 50D than the shoot portions of germinating *P. radiata* seeds. There was an apparent increase in the cytoplasmic-RNA content of these tissues, particularly in cells associated with a layer of proliferating tissue surrounding the vascular cylinder. Root tissues of embryos of the control treatment contained a relatively limited region of small-diameter cells with dense cytoplasm and a high RNA content, behind the root apex. The comparable zone in the herbicide-treated root tissues was much larger due to massive cell proliferation and suppressed cell elongation. Hence it would appear that the cytoplasmic-RNA content was greater in the herbicide-treated than in the control root tissues. However, without further detailed investigations it is extremely difficult to determine whether this RNA increase is on a cellular basis. Accompanying the apparent increase in RNA, there was a significant increase in the volume of the nucleolus in cells of herbicide-treated root tissues compared with those of the control. Tordon 50D affected nucleoli volume more than did Tordon 22K. This increase in size may be due to physical swelling or may indicate

stimulated activity associated with the nucleolus. Prominent nucleoli were also observed in vascular cells of eucalypt tissues treated with picloram and 2,4-D (Chapter 7). Similarly, Nitsch (1968) has shown that IAA treatment caused swelling and increased vacuolation of nucleoli in tissue culture cells. In this study, the increase in volume of the nucleolus may indicate an increase in DNA content of the cells resulting in DNA-dependant RNA synthesis in the root.

The concept that auxins and auxin herbicides stimulate RNA synthesis in plant tissues is widely accepted. The effects of auxin herbicides on cell division and the subsequent proliferation of a disorganized body of rapidly and irregularly dividing cells leading to swelling of root tissues has been attributed directly to unregulated and excessive synthesis of cytoplasmic-RNA (West et al. 1960, Key and Hanson 1961, Chrispeels and Hanson 1962). Fites et al. (1969) found increased cytoplasmic-RNA synthesis in herbicide-treated soybean hypocotyls. This was preceded by an increased production of DNA. RNA associated with the nucleus, presumably messenger-RNA, was also markedly stimulated and active proliferation of certain stem cells resulted.

The normal regulation of RNA synthesis may be a basic function of auxin action and is associated with processes of cell division and cell elongation (Key and Ingle 1968). Messenger-RNA in particular is concerned with cell expansion, and Key and Ingle (1968) suggested that messenger-RNA couples with pre-existing ribosomes of the endoplasmic reticulum to mediate protein synthesis essential for further cell development. Masuda (1968) also reported that the auxins, indole-3-acetic acid (IAA) and 2,4-D, stimulated RNA synthesis, possibly messenger-RNA, which in turn produced an enzyme responsible for cell wall loosening and subsequent cell expansion.

Davidson and Webster (1968) suggested that auxin (IAA) may affect DNA synthesis and thus regulate mitotic cycles. A close association between auxin activity and nucleic acid synthesis affecting cell division has been shown by other workers. Masuda et al. (1966) noted that the stimulation of cell division by IAA was checked in tissues treated with actinomycin D, an inhibitor of DNA-dependent RNA synthesis. Klein (1968) used ultraviolet radiation to block the synthesis of RNA and protein in rice coleoptile segments and cells were unable to respond to IAA treatment. Klein also found that growth processes controlled by other growth regulators such as the gibberellins and cytokinins remained unaffected by radiation treatment.

A strong relationship appears to exist between auxin activity and nucleic acid synthesis leading to the growth of the cell. However, other growth hormones are also essential for cell development and it is unlikely that auxin alone is responsible for the activation of any one specific cell process. Fosket and Torrey (1969) reported that the auxin, naphthalene-acetic acid (NAA), at concentrations of  $10^{-7}$  to  $10^{-5}M$  stimulated cell division and subsequent cell differentiation in soybean tissue cultures but only in the presence of an effective concentration of kinetin. 2,4-D at  $10^{-7}$  and  $10^{-6}M$  concentrations promoted both cell proliferation and tracheary element differentiation in the absence of an exogenous kinetin supply (Fosket and Torrey 1969). Treatment of tobacco tissue cultures with auxin (IAA) alone caused cell enlargement but not cell division (Nitsch 1968). The cultures gave no growth response when kinetin alone was used but treatment with kinetin, preceding the addition of auxin stimulated cell division. Nitsch (1968) observed that the conditioning effect of kinetin in the cells, prior to auxin stimulation, was linked with DNA and messenger-RNA synthesis. Gibberellic acid was unable to



replace kinetin in stimulating cell proliferation but, when added with kinetin, a synergistic effect was observed. The activity of both kinetin and gibberellic acid involved active DNA synthesis.

Growth hormones in animal tissues also stimulate RNA synthesis (Tata 1970). In rat liver tissue and seminal vesicles an abrupt increase in RNA and protein synthesis of the endoplasmic reticulum followed treatment with labelled growth hormone. This increased synthesis gave rise to an increase in the production of phospholipids associated with cell membranes. Tata (1970) suggested that the stimulation of nucleic acid synthesis by growth regulators was also responsible for the formation of membranes, particularly those of the endoplasmic reticulum where rapid accumulation of membrane phospholipids occurred.

The evidence in the literature suggests that the relationship between growth regulators, auxins in particular, and cell division, cell elongation, and growth in general, is through nucleic acid synthesis. In the current study root tissues of P. radiata embryos, treated with the auxin herbicides Tordon 22K and Tordon 50D, appeared to possess greater amounts of cytoplasmic-RNA than did root tissues of the control treatment. Furthermore, nucleoli were larger in cells of the herbicide-treated than in the control tissues, perhaps an indication of an effect on DNA-synthesis. These herbicide-induced disruptions in the normal cell regulation of nucleic acid synthesis could have been responsible for many of the growth irregularities observed in the root tissues, particularly the effects on cell division and elongation.

Other responses to the Tordon herbicides, notably the very rapid effect on the integrity of the cell membranes and the disruption in the normal plane of cell division, are not

readily explained in terms of altered nucleic acid synthesis.

Ginzburg and Kende (1968) found that of all the subcellular fractions examined in dwarf pea tissues treated with labelled gibberellic acid, only membrane components showed any radioactivity. They were unable to determine if the attachment of the growth regulator to the membrane surface was of any physiological importance but it did suggest that gibberellic acid could act on membrane permeability or in the regulation of metabolic processes occurring on or in the membrane surface. Collins and co-workers (1971) investigated soluble nucleotides in wheat grain aleurone tissue treated with gibberellic acid to determine to what extent RNA metabolism was altered by the hormone. They found that gibberellic acid had no appreciable effect on the rate of turnover of three precursors of nucleic acid synthesis and concluded that the hormone did not exert a primary effect on RNA synthesis. However, the hormone markedly stimulated the rate of turnover of cytosine triphosphate (CTP), a substance directly concerned with the synthesis of phospholipids essential for cell membrane construction. Hence they concluded that the increase in CTP activity after treatment with gibberellic acid was a manifestation of the effect of this hormone on membrane synthesis.

Auxins have also been shown to affect cell membranes. Andreae (1967) proposed that the growth inhibition of pea root segments treated with exogenous auxins originated at a site external to the cytoplasm, possibly the cell wall or the plasmalemma. Cocking (1968) suggested the plasmalemma as one cellular site of auxin activity. He showed that isolated tomato fruit protoplasts, free of cell walls, responded to IAA ( $10^{-6}$ M) by producing numerous outgrowths from the surface of the plasmalemma but not from the tonoplast. These outgrowths were apparently connected with some process in cell wall regeneration.

Nitsch (1968) also demonstrated a membrane response to IAA treatment by protoplasts of tobacco callus cells in culture. Invaginations (pinocytes) were formed by the membranes after treatment and it was suggested that this may be the mechanism by which water uptake into plant cells is stimulated by auxin treatment. Pinocytosis may also play an important role in active ion uptake by cells (Hall 1970, Wheeler and Hanchey 1971) but this is only one of several ways in which substances can move across membranes (Hogben 1962).

Permeability of cells can be increased by low auxin concentrations and decreased at higher, growth-inhibiting levels (Kramer 1955). Sacher (1959) noted that 4ppm IAA directly influenced the semi-permeability of cell membranes and that growth-promoting levels of 2,4-D also increased the uptake of uncharged particles in tissues, apparently by affecting the property of the membrane controlling the movement of these particles. Permeability and hence uptake of solutes decreased with higher 2,4-D concentrations, due possibly to changes on the surface of the membranes. Sacher (1959) concluded that a primary function of endogenous auxin must be in the maintenance of membrane properties. He observed that without an exogenous supply of auxin, tissue segments of in vitro cultures rapidly became soft and flaccid with movement of solutes and water out of the protoplasts into the intercellular spaces. Wedding et al. (1959) found that  $10^{-2}M$  2,4-D decreased membrane permeability to sucrose and manitol ions. They suggested a number of auxin effects on growth might be due, in part, to changes in the permeability of membranes to solutes. However, Kang and Burg (1971) questioned whether or not the changes in membrane permeability of excised pea stems by IAA was casually related to auxin-induced growth. They observed that the movement of water into and out of the tissues did not exactly parallel the growth response to IAA. Van

Steveninck (1965) found a wide range of IAA concentrations induced increased leakage of potassium ions from young beetroot tissue and that ageing of the tissue reduced the sensitivity to auxin. Van Steveninck suggested that a primary action of auxin was in effecting changes in membrane permeability, particularly in young, receptive cells.

In the current study, the earliest observed response of P. radiata needle segments treated with Tordon 50D was a marked increase in membrane permeability as measured by the alternating-current bridge (Chapter 5). This response was noted within two hours of treatment, and after four hours acute membrane disruption and death of the tissues was apparent. Tordon 22K had no effect on the membrane permeability of cells of P. radiata needle segments. This was the first marked indication of a differential response to the Tordon herbicides.

Plasma membranes of E. viminalis leaf discs were affected by the concentrations of both Tordon 50D and Tordon 22K used. The breakdown of the tonoplast and internal cytoplasmic membranes in some cells destroyed the ordered peripheral distribution of the chloroplasts, and enabled them to move and clump together within the cells. Plasmalemma permeability properties were also altered, the permeability increasing within 24 hours after treatment with Tordon 50D, and after 72 hours with Tordon 22K (Chapter 5).

Tordon 50D-treated needle tissues examined under the electron microscope showed an early stage of disruption of cell integrity was the gradual withdrawal of the plasmalemma from the cell wall (Chapter 6). With longer periods of herbicide treatment, the plasmalemma became greatly distorted, cytoplasm disappeared, and the nucleus gradually disintegrated. Similar effects on the cell membranes of P. radiata needle segments by Tordon 50D and of E. viminalis leaf discs by both Tordon 50D and Tordon 22K were again observed in treated intact seedlings of P. radiata and E. camaldulensis (Chapter 7).

Chloroplast membranes were also markedly affected by herbicide treatment. Tordon 22K and Tordon 50D had similar effects on pine chloroplasts although the effect of the former was relatively delayed. The initial response of both pine and eucalypt chloroplasts to herbicide treatment was an overall swelling, possibly due to changes in permeability of the bounding chloroplast membrane permitting the plastids to increase in size without rupturing. As treatment continued, the normally flattened thylakoid membranes of the grana and the fretwork systems also became swollen, the parallel alignment of thylakoid discs was greatly disrupted, and large spaces were created between adjacent membranes. The volume of individual thylakoid discs increased without any rupture or decomposition of the bounding membrane, ultimately causing the complete disarray of the previously ordered granal stacking. It is possible that herbicide molecules altered forces existing on the surface of the internal membranes causing them to stretch and move out of their normal alignment. Brian (1964) notes that changes in surface membrane properties have been caused by forces of attraction set up between the membrane surface and the non-polar portions of auxin herbicide molecules. The degree of penetration of the herbicide molecule depended on the structure of its non-polar portion. Erickson *et al.* (1955) noted that in cells of Chlorella treated with 2,4-D, only undissociated, uncharged herbicide molecules were able to penetrate the membrane.

Intermolecular forces at the surface of the plasmalemma are probably important in controlling the penetration of molecules into the cytoplasm. For example, Smith (1970) suggests that the accumulation of mineral ions by plant cells is accompanied at the membrane surface by the movement of protons ( $H^+$ ) and hydroxyl ions ( $OH^-$ ). He demonstrated that

chloride ion transport across the membrane could be regulated by changing the pH of the bathing solution and that this depended on an initial separation of  $H^+$  and  $OH^-$  at the surface of the plasmalemma.

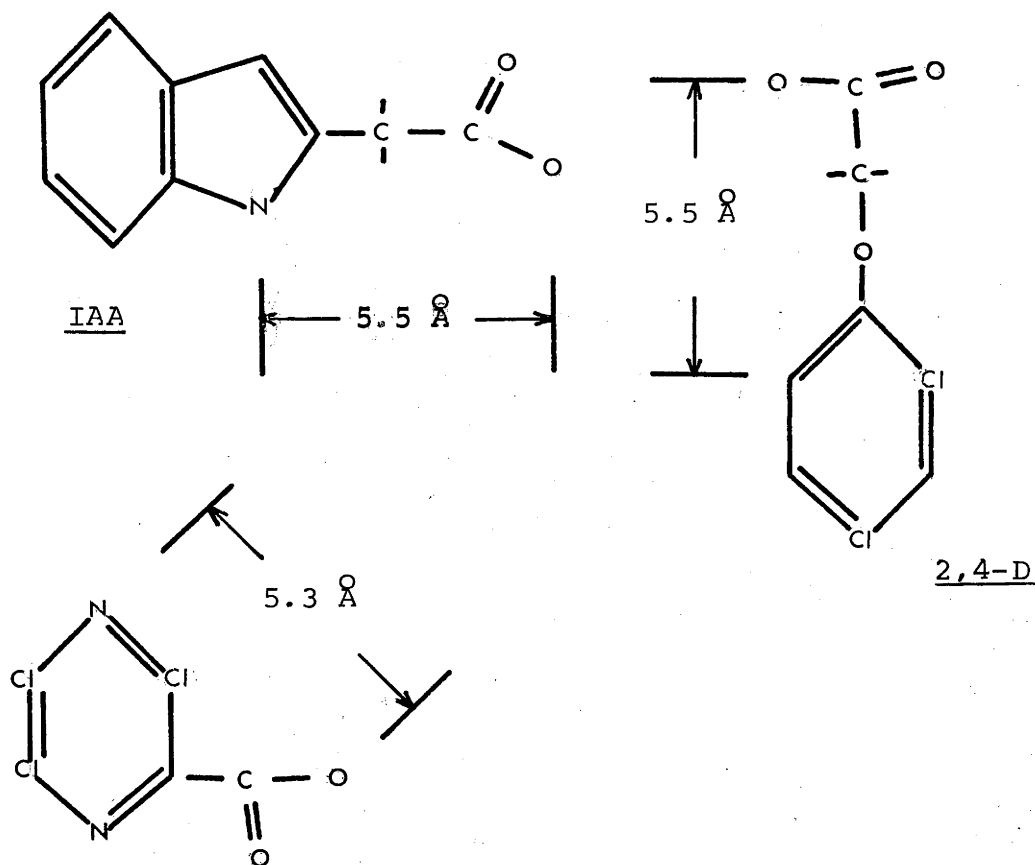
The size of the molecules as well as their lipid solubility influences their penetration (Collander 1957). Membranes are composed of globular protein macromolecules, the spaces between adjacent proteins being filled with lipid chains. These lipid chains are structural lipids such as the phospholipids, glycolipids, isoprenoids and the sulpholipids, important for stabilizing the membrane and regulating its permeability properties (Thompson 1965). Many protein and lipid subunits in a specific membrane are bound together by forces of attraction, the nature and strength of these forces depending upon the types of amino acids present in the protein macromolecule. Water also forms a system of interlocking H bonds with the polar groups of the lipid and protein molecules. Thompson (1965) suggests that the strongest forces on the membrane surface are electrostatic forces produced by ionized side chains of one molecule attracted to oppositely charged regions of another nearby molecule.

Reports in the literature and observations made in this study of Tordon herbicide activity on plant tissues suggest the surface of the plasmalemma as a possible primary site for auxin herbicides to exert their toxicity. This could be brought about by changing the forces on or at the membrane surface, thus altering its permeability to essential solutes. Changes in permeability may lead to changes within the cytoplasm, bringing about irregularities of growth. There is also the possibility the herbicides have a direct effect on internal cell membranes, notably the chloroplast membrane and to some extent, the endoplasmic reticulum.

In view of these possibilities it is interesting to note van Overbeek's theory on auxin action (van Overbeek 1961) first proposed at the Fourth International Conference on Plant Growth Regulators held in 1959. Van Overbeek suggested that auxin affects the activity of many metabolic enzymes, possibly at the membrane surface upon or in which the enzymes are situated. The contact of the auxin molecule could lead to a local change in the hydration of the membrane, changing the relative distance between the enzymes on or in the surface. This would affect their relative reaction rates, ultimately upsetting the balance of metabolites, and consequently affect the physiology of the cell. At the surface of the membrane, the auxin molecule moves into a cavity, is anchored there by the ring portion of the molecule, and has its polar side chain protruding above the membrane surface. This polar group, according to van Overbeek, becomes part of an H-bond system at the surface, and sets the system oscillating. The strengthening of the already existing H-bond system (its existence has also been suggested by Thompson (1965) and by Smith (1970)) by polar groups of the auxin molecule would affect the structure of the membrane, causing it to contract or, by hydration, to expand. The polar side chain becomes the critical portion of the auxin molecule, the remainder being necessary for its exact placement at the membrane surface among the H-bond system, i.e. the ring structure serves to anchor the molecule in the membrane. Van Overbeek concluded by suggesting that the primary auxin function is in the participation of the undissociated acid group in a hydrogen bond system at the membrane surface and to set it oscillating.

The structural composition of both 2,4-D and picloram would appear to satisfy van Overbeek's requirements for substances to act at or on the membrane surface. For auxins

to act in such a way one could well imagine a critical spatial requirement in molecules exhibiting auxin activity. Porter and Thimann (Thimann 1963) have proposed, the critical property for auxin activity is a distance of approximately  $5.5 \text{ \AA}$  between the carbon of the carboxyl and a fractional positive charge in the nucleus (compare the structural diagram of IAA and those of 2,4-D and picloram).



Picloram (Kefford and Caso 1966)

The possible effect of auxin herbicides on the membrane surface does not explain the unregulated cell growth observed in root tissues of P. radiata seeds treated with Tordon 22K and Tordon 50D. It nominates the possible primary site of herbicide action but not how this site is related to aberrant cell division and subsequent tissue proliferation.



Weintraub (1953) observed that auxin controls the ordering of normal cell division and suggested that one 2,4-D molecule may antagonize approximately 80 molecules of IAA, bringing about unpolarized cell division, abnormal growth and metabolic and physiological disturbances.

The plane of cell division is thought to be determined by factors in the cytoplasm, and Sinnott (1960) states that, prior to cell division, a cytoplasmic diaphragm stretches across the cell in the position where the future cell wall will be formed. In these dividing cells, the direction of the axis may be related to gradients in hormone concentration or balance in the cytoplasm (Sinnott 1960). It is possible that alterations of the membrane surface changing the permeability of the membrane to solutes, can cause changes in this hormone gradient and give rise to growth irregularities.

In germinating P. radiata seeds treated with Tordon 22K and Tordon 50D, apparent changes in the normal plane of cell division of cells immediately below the promeristematic region were associated with herbicide treatment. In addition to normal transverse divisions, frequent irregular longitudinal and oblique planes of division were observed and are responsible for the abnormal swelling of the epicotyl. Root tissues showed a pronounced response to herbicide treatment and changes in the plane of cell division were most obvious here. Cells in and around the vascular region failed to elongate and divided abnormally, creating a vast proliferation of small cells which continued to grow irregularly, increasing the thickness of the root. This aberrant, apparently unregulated cell division caused the crushing of cells external to it and possibly disrupted the functioning of the vascular tissues. Cell proliferation was also observed in intact P. radiata seedlings treated with Tordon 50D.

Sinnott (1960) reports that proliferating cells in crown gall tumors produced by the bacterium, Agrobacterium

tumefaciens, do not require an external source of auxin for continued, unregulated growth. He suggests that the process of converting normal cells into tumor cells may result from the acquirement by the tumor cells of the capacity to synthesize auxin. In Nicotiana plants, Ames et al. (1969) found that IAA application prevented or suppressed the formation of stem tumors. They noted however, that the method of transforming normal cells into tumorous cell growth is not understood.

Recent reports concerning the growth of animal tumor cells suggest a possible explanation and may also help to explain the unregulated cell growth in P. radiata tissues treated with the Tordon herbicides.

Loewenstein (1970) describes a kind of cellular communication ideally suited for controlling the arrangement of cells in a developing organism. This communication is brought about through permeable membrane junctions which are organized to form passageways between cells. The communication between animal cells in normal growth is brought about by a conversion in permeability of these membrane junctions owing to changes in the concentration of calcium ions. Permeability is high only when the concentration of calcium ions in the cell cytoplasm is low relative to that outside the cell. Loewenstein (1970) assumed that certain binding sites on the membrane surface occupied by calcium ions in the impermeable state, are unoccupied in the permeable state. Rasmussen (1969) also notes that calcium is a key component of the membrane in animal cells and changes in calcium binding on the surface can alter many physical properties of the membrane, for example, its permeability to water and to other ions.

Loewenstein (1970) suggests that the range in the size of particles permeating the membrane junctions is wide enough to include most molecules involved in metabolism (up to a molecular weight of about 10,000), and many other molecules that regulate cellular activities. Thus, possibly the junction is instrumental in conveying substances controlling the growth and differentiation of cells. Communication may be confined to young developing cells, being lost when the cells are fully differentiated.

If junctional communication is a gateway for growth-controlling substances unregulated cell growth could be due to poor junctional communication.

Work with tumors from mammalian liver, thyroid in the rat, and human stomach epithelium indicates that lack of junctional communication is a manifestation of cancerous growth but not of growth in general. However, Loewenstein (1970) concludes that the observed defects in junctional communication between cells is probably not the only cause of unregulated cell growth.

Other workers in cancer research have reported that changes to the membrane surface of cells may result in aberrant tissue growth.

Aaronson and Todaro (1968) found that tumors were produced in mouse tissues by contact-insensitive cells. Normal cells in these tissues have a built-in control mechanism that prevents any further growth beyond a certain stage. Tumors developed when normal cells lost this control mechanism and continued to grow and divide. Pollack and Burger (1969) assumed that changes on the outer surface of the membrane, possibly caused by a tumor virus, transformed normal cells to cells lacking contact-inhibition. They found a close relationship between the availability of certain agglutinin receptor sites on the membrane surface and the loss of the contact-inhibition control mechanism. Proteases such as trypsin dissolved the outer layer of the membrane, exposing

these receptor sites and cells lost their contact inhibition of cell division. In further work, Burger and Noonan (1970) covered these receptor sites by replacing the outer layer of the surface membrane with a plant protein, an agglutinating agent, concanavalin A and growth control was immediately restored.

It is clear, therefore, that surface membrane properties may play an extremely important part in the growth of animal cells. Extrapolating these observations on animal cancer cells to the unregulated cell growth in plant tumors and to the mass of proliferating cells in P. radiata root tissues treated with the Tordon herbicides, is possibly questionable. Sinnott (1960) notes, malignancy is difficult to define in the same terms in organisms as different in structure and organization as plants and animals. However, Cook (1971) points out that it is often advantageous to consider animal membrane systems, for some of the approaches adopted for animal systems may have wider biological significance. Furthermore, one at least has an hypothesis from which to reason, plan and experiment.

In this current study, there are two important herbicide-induced effects that must be emphasized. Firstly, the 2,4-D component of Tordon 50D rapidly disrupted cell membranes in P. radiata, and in eucalypt tissues. Picloram although much less effective in P. radiata, was similar to 2,4-D in its action on eucalypt tissues. Secondly, both 2,4-D and picloram disrupted the normally ordered growth in meristematic regions of P. radiata seeds and seedlings.

Both these responses may be explained in terms of direct effects of the herbicides on plant cell membranes. The breakdown of P. radiata cell membranes by Tordon 50D (and 2,4-D) was very rapid and was observed within two hours of treatment, indicating a direct herbicide effect on the membranes

of these cells. Although the action of herbicides on the eucalypt tissues was slower, a direct effect of the herbicides on cellular membranes is again possible.

While recognizing Sinnott's (1960) objection to animal and plant comparisons, a parallel can be tentatively drawn between the cancerous growth of some animal cells and the disruption by the Tordon herbicides of the ordered and regulated growth of actively growing meristematic cells. If it is assumed that the growth of plant cells is controlled by membrane properties, as appears the case in animal cells, then even minor modifications of the membrane by herbicides could result in growth disturbances. Such effects would appear more likely in actively growing, undifferentiated cells than in fully formed cells. Differences in reaction of P. radiata tissues to Tordon 22K and Tordon 50D could be explained by the following hypothesis. Both picloram and 2,4-D affect the membranes of meristematic cells sufficiently to cause growth abnormalities, however only 2,4-D (an active component of Tordon 50D) has sufficient effect on the membranes of older, more differentiated cells to disrupt these completely. Response to auxin herbicides can probably be modified by the growth stage of the tissue and the progress of metabolic changes associated with ageing. Ageing reduced the sensitivity of tissues to applied IAA (Van Steveninck 1965). Apart from other changes in membrane characteristics, changes in permeability properties occur as meristematic cells develop and mature (Laites 1964). This may also help to explain the greater effect of Tordon 50D on P. radiata tissues compared with Tordon 22K.

The differential effect of Tordon 22K on pine and eucalypt tissues is not readily accounted for in terms of a membrane effect. Although chloroplast structure was affected

in needle and leaf disc material treated with Tordon 22K, only in eucalypt tissue was there an effect on the plasmalemma. Whether this latter contrast represents differences in structure or membrane properties between the pine and eucalypt material or is a manifestation of other factors such as herbicide penetration, is beyond the scope of this present study.

Additional observations of auxin activity in the literature could also be explained in terms of membrane properties. In particular, the effects of calcium, and other metallic ions, as well as chelating agents, in modifying the growth of plant sections (Thimann and Takakashi 1961) could conceivably be due to effects on ionic gradients between the interior and exterior of the cell (cf. Loewenstein 1970).

Changes in solute traffic across plant membranes is related to metabolic changes and also to changes in the permeability of the membranes (Laites 1964). Higinbotham et al. (1970) recently reported that cyanide and dinitrophenol rapidly depolarize cells of oat coleoptiles and pea epicotyls. Active ion transport across the cell membrane was blocked possibly due to an inhibition of metabolism, particularly in adenosine triphosphate synthesis (ATP). The site of the ion pump was thought to be in the plasmalemma and the depolarization effect of cyanide and dinitrophenol could be reversed only if the permeability of the membrane was not impaired.

In this discussion the controlling influence of cell membranes on the growth of animal cells has been highlighted and it is important to recognize that the walls of plant cells prevent the intimate association of plant cell membranes possible in animal cells. Nevertheless, the protoplasts of individual plant cells are in contact with one another via plasmodesmata allowing similar forms of control mechanisms.

Proposals that auxins act directly on plant cell membranes are by no means new but, generally, the conclusion that this is a primary or even an important aspect of auxin action has been discounted (Galston and Purves 1960). More

recent proposals of the very subtle control which cell membranes can exert on cell growth, however, suggest the whole concept of the role of cell membranes in plant growth requires careful investigation.

Auxins and auxin herbicides certainly affect nucleic acid metabolism but even this could be a secondary effect resulting from changes in the membranes. Much of the metabolism of plant cells is intimately associated with membranes - internal membranes such as the endoplasmic reticulum, membranes associated with cell organelles, as well as the plasma membranes - consequently change in membrane structure could influence a very wide array of metabolic reactions.

The work described in this study has not fully explained the differential effects of the Tordon herbicides on pine and eucalypts. On the other hand, it has shown that auxin herbicides can act directly on plant cell membranes and suggests this is an important consideration in explaining the action of auxin herbicides. Evidence from the literature, particularly that dealing with animal tissues, supports such a possibility. This area of research is exciting in its potential.

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